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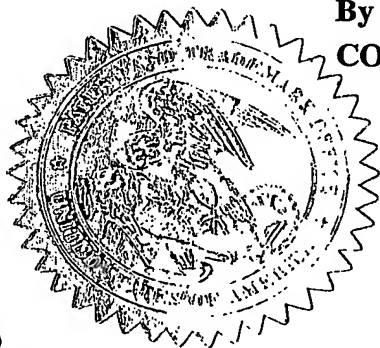
APPLICATION NUMBER: 60/421,112

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Patent

Attorney's Docket No. 017753-167

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

UTILITY PATENT
APPLICATION TRANSMITTAL LETTER

Box PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Enclosed for filing is the utility patent application of DIDIER HOARAU, PASCAL DELMAS and JEAN-CHRISTOPHE LEROUX for STEALTH LIPID NANOCAPSULES, METHODS FOR THE PREPARATION THEREOF AND USE THEREOF AS A CARRIER FOR ACTIVE PRINCIPLE(S).

☐ Applicant(s) hereby request(s) that the above-captioned application **NOT BE PUBLISHED** under 35 U.S.C. § 122(b) and 37 C.F.R. § 1.211. The undersigned hereby certifies that the invention disclosed in this application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

☒ Applicant(s) suggest(s) Figure 1 for inclusion on the front page of the patent application publication and patent.

Also enclosed are:

☒ 3 sheet(s) of drawings;

☒ a claim for foreign priority under 35 U.S.C. §§ 119 and/or 365 is ☒ hereby made to 02/07175 filed in FRANCE on JUNE 11, 2002;

☒ in the declaration;

☐ a certified copy of the priority document;

☐ a General Authorization for Petitions for Extensions of Time and Payment of Fees;

☐ an Assignment document;

☐ an Information Disclosure Statement;

☐ a patent application data sheet; and

☐ Other: _____

☒ An ☐ executed ☒ unexecuted declaration of the inventor(s)

☒ also is enclosed ☐ will follow.

☐ Small entity status is hereby claimed.

☒ Please amend the specification by inserting before the first line the sentence:

--This application claims priority under 35 U.S.C. §§ 119 and/or 365 to 02/07175 filed in FRANCE on JUNE 11, 2002; the entire content of which is hereby incorporated by reference.--

☐ Please amend the specification by inserting before the first line the sentence:



21839

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09/09/02
1129 U.S. PTO

10951 U.S. PTO
60/421112
09/09/02

Utility Patent Application Transmittal Letter
 Attorney's Docket No. 017753-167
 Page 2

--This application claims priority under 35 U.S.C. § 119 to U.S. Provisional Application No. entitled and filed on , the entire content of which is hereby incorporated by reference.--

☐ Please amend the specification by inserting before the first line the sentence:

--This application is a continuation of International Application No. filed on --.

☒ The filing fee has been calculated as follows ☐ and in accordance with the enclosed preliminary amendment:

CLAIMS					
	NO. OF CLAIMS		EXTRA CLAIMS	RATE	FEE
Basic Application Fee					\$740.00 (101)
Total Claims	67	MINUS 20 =	47	× \$18.00 (103) =	\$846.00
Independent Claims	1	MINUS 3 =	0	× \$84.00 (102) =	-0-
If multiple dependent claims are presented, add \$280.00 (104)					\$280.00
Total Application Fee					\$1,866.00
If small entity status is claimed, subtract 50% of Total Application Fee					
Add Assignment Recording Fee \$40.00 (581) if Assignment document is enclosed					
TOTAL APPLICATION FEE DUE					\$1,866.00

☐ This application is being filed without a filing fee. Issuance of a Notice to File Missing Parts of Application is respectfully requested.

☒ A check in the amount of \$ 1,866.00 is enclosed for the fee due.

☐ Charge \$ to Deposit Account No. 02-4800 for the fee due.

☒ The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. This paper is submitted in duplicate.

Please address all correspondence concerning the present application to:

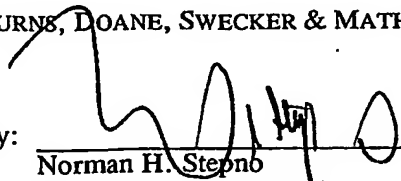
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Respectfully submitted,

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Date: SEPTEMBER 9, 2002

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The subject of the present invention relates to nanocapsules which, as a carrier for active principle(s), can be used for the treatment of solid or circulating cancerous tumors, which exhibit stealth properties with respect to the immune system of the host to which they are administered and which are capable not only of undergoing extravasation into the tumor, but also of releasing their content therein. This novel carrier in accordance with the invention is advantageously in the form of an injectable colloidal suspension of lipid nanocapsules carrying at their surface phospholipid molecules associated with poly(ethylene glycol) molecules.

The present invention also relates to the method for synthesizing these stealth lipid nanocapsules, and in particular the method for inserting said molecules carrying poly(ethylene glycol) groups into the lipid envelope of the preformed nanocapsules.

Finally, this invention relates at once to the uses which can be made of such a carrier, for delivering active principles via the blood, in particular for the treatment of solid or circulating tumors in humans, but also for the treatment of inflammations or of any other pathological condition for which an increase in vascular permeability is observed (inflammatory areas, infectious sites).

The present invention also relates to the various pharmaceutical compositions comprising the nanocapsules according to the present invention.

First of all, the term "carrier" is intended to mean any chemical entity which allows the transport and delivery, in the organism to which it is administered, of molecules, and in particular of active principles, such as medicinal products for example.

Moreover, the term "colloidal" is intended to mean any stable dispersion of solid elements with a size of less than 1 micrometer in a liquid phase.

The term "stealth" used to describe the carrier in accordance with the present invention denotes the ability of the nanocapsules not to be detected and then sequestered and/or degraded, or to be hardly detected and then sequestered and/or degraded, and/or to be detected and then sequestered and/or degraded late, by the immune system of the host to which they are administered.

The term "extravasation" is used to denote the exit of a body from the blood compartment. This exit takes place through pores or "fenestrations", tangentially to the axis of the blood capillary. This phenomenon is only observed in certain organs, the capillaries of which are fenestrated, such as the kidney or the liver, for example.

and in particular extracerebral solid tumors.

The term "nanocapsules" is intended to mean spheres with a diameter of less than 300 nm comprising a circular wall more or less rigid at ambient temperature and a central component or core which, in the case of the present invention, contains fatty substances.

The term "amphiphilic" is used to describe a molecule carrying groups having an affinity for substances which are hydrophobic in nature and groups having an affinity for substances which are hydrophilic in nature.

Finally, "mol%" expresses a molar percentage and the term "active principle" denotes a pharmaceutically active principle.

The stealth lipid nanocapsules which are the subject of the present invention consist of an essentially lipid core which is liquid or semi-liquid at ambient temperature, and an outer lipid envelope comprising at least one hydrophilic surfactant which is lipid in nature, at least one lipophilic surfactant which is lipid in nature and at least one amphiphilic derivative of poly(ethylene glycol) (PEG), the molar mass of the poly(ethylene glycol) component of which is greater than or equal to 1 000 g/mol, preferably greater than or equal to 2 000 g/mol.

The "pegylated" amphiphilic derivative contributes to conferring the stealth aspect on the nanocapsules. The core of the nanocapsules advantageously allows the incorporation and transport of many molecules, and more particularly of active principles which are lipophilic in nature, such as anticancer agents, transported in dissolved or dispersed form. The nanocapsules in accordance with the invention are thus capable of carrying anticancer medicinal products in the blood system, and more particularly in the interstitial medium bordering tumors, for sustained periods of time. Moreover, the nanocapsules in accordance with the invention have a sufficiently long blood circulation time, allowing them to undergo interactions with circulating tumor cells and greater extravasation in solid tumors.

"Solid" tumors consist of tumor cells organized in solid clumps or in tissues irrigated by the blood circulation. Many tumors are irrigated by a network of fenestrated capillaries. These capillaries thus have types of pores which, depending on their size, allow solid elements to pass out of the blood compartment. The size of the fenestrations between the endothelial cells of the basal membrane of tumor vessels varies from one tumor to another. However, colloidal particles less than 300 nm in size can undergo extravasation in some tumors. This permeating of

colloidal particles to the interstitial medium of the tumor is, moreover, combined with an increased retention effect due to poor lymphatic drainage of the tumor. This double phenomenon, named EPR for *Enhanced Permeability and Retention*, has made it possible to envisage the development of an antitumor therapy based on colloidal carrier systems. Specifically, EPR allows significantly sustained accumulation, compared to that which is observed on healthy tissues, of colloidal particles and of macromolecules in the area around the tumor.

This EPR phenomenon has thus been exploited in order to form colloidal carriers intended to transport anticancer active principles. However, in order to be truly effective, such carriers must be able to circulate in the blood compartment, for a sufficient amount of time before being detected and then destroyed by the host's immune system. Thus, non-stealth colloidal objects are rapidly detected by certain effectors of the immune system, in particular such as macrophages, and cannot reach tumors in sufficient amounts to be effective.

In fact, macrophages constitute one of the most important components of the immune system and play a predominant role in eliminating foreign particles from the blood circulation, including liposomes and other colloidal particles.

The recognized expression for denoting this set of circulating and sedentary monocytes which constitute the non-specific defence system involved in capturing nanoparticles is "the Mononuclear Phagocyte System" (MPS).

At the molecular level, the clearance of injected particles takes place in two steps: opsonization by the depositing of serum proteins (or "opsonins") at the surface of the particles followed by recognition and capture of the opsonized particles by macrophages.

Modification of the surface of nanoparticles with chains of hydrophilic and flexible polymers, generally of the poly(ethylene glycol) type, confers them a steric protection by preventing the opsonins reaching the surface of the particles and bringing about the elimination thereof by the cells of the MPS. The effectiveness of these polymer chains in conferring a stealth nature depends both on their length and on their density. Thus, Mori *et al.* (FEBS Lett., 284: 263, 1991) have carried out comparative studies with phospholipids comprising chains of PEG 750, 2 000 and 5 000 g/mol, present at the surface of 200 nm liposomes. At equal molar percentage, PEG 750 proved to be completely ineffective *in vitro* in conferring steric

protection against opsonization. On the other hand, the longest PEG chains exhibit an effectiveness against opsonization which is related to their length. This *in vitro* behaviour is correlated with the half-lives of the liposomes, after intravenous injection into mice, which are: less than 30 minutes, approximately 2 hours and more than 3 hours for PEG 750, PEG 2000 and PEG 5000, respectively.

Other studies (Allen *et al.*, BBA, 1066: 29, 1991) demonstrated that, below 1 000 g/mol, the poly(ethylene glycol) chains, even when they are at saturating concentration at the surface of the nanoparticles, do not allow them to confer a sufficient steric protection and therefore stealth properties.

The design of stealth carriers intended to transport anticancer active principles makes it possible not only to increase their therapeutic effect, but also to thus ensure a certain invisibility with respect to the opsonization phenomenon and the immune system. Moreover, this increase in their blood half-life time (consequence of the stealth effect) may also serve to decrease the toxicity of the carried active principles, which will mainly be released in the interstitial tissue of the tumor with very limited diffusion in healthy tissues.

In fact, anticancer active principles are generally molecules which are small in size and which exhibit a high cytotoxicity, particularly pronounced on dividing cells. Moreover, the effective therapeutic concentration thereof is sometimes not very different from the plasma concentration at which they become toxic for the organism to which they are administered. This narrow concentration window requires the use of carriers for the administration of such compounds. The use of stealth carriers allows them to accumulate in the interstitial tissue of tumors and to release their active agents therein. Due to this accumulation, the intratumoral concentration of active principle is very high, much greater than the concentration which can be reached using conventional carriers or with the active principle alone (Allen, Trends, Pharm. Sci., 15: 215, 1994). Stealth colloidal systems therefore make it possible to use higher doses of anticancer agents, greater than those which it is possible to administer with the active principles alone due to their high toxicity.

The accumulation of nanoparticles in tumors and the resulting therapeutic effect depends on the half-lives of their elimination from the blood circulation. Thus, various formulations of liposomes of the conventional type or exhibiting sustained circulation have been compared on the basis of their blood elimination profile and of their accumulation in tumors in mice (Gabizon A. and Papahadjopoulos D., Proc.

Natl. Acad. Sci. USA, 85: 6949, 1988). Thus, a stealth formulation having a half-life of approximately 8 hours shows an accumulation of more than 10% of the injected dose in the tumors after 24 hours. Control formulations having half-lives of slightly less than one hour show accumulations in tumors which are much lower than that observed for the stealth formulation. Specifically, the intratumoral concentrations of the control formulations, at 24 h, represent only 4 to 16% of the amount assayed in the tumors in the case of the stealth formulation. These results show that formulations having half-lives of the order of one hour or less than an hour accumulate poorly in tumors.

Colloidal systems such as liposomes or nanoparticles, i.e. consisting of particles in suspension which are 25 nm to 1 μ m in diameter, led to the development and the commercialisation of various colloidal pharmaceutical forms.

Thus, US patent No. 5,811,119 describes the use of liposomes for administration of anticancer medicinal products derived from carotenoids. This liposomal formulation in particular makes it possible to decrease the *in vivo* toxicity of these active principles. The carotenoids intended to treat tumors are here combined with an intercalation-promoting agent which allows their loading in the liposome. The transport of these lipophilic active principles in a liposome, the core of which is hydrophilic in nature, is possible due to the presence of amphiphilic compounds such as triglycerides. These "intercalation-promoting agents" make it possible to transport carotenoids in a ratio of 10 mol of triglycerides to one mole of carotenoid.

Such a carrier makes it possible to envisage transporting anticancer agents while decreasing their cytotoxicity, but has a low encapsulation rate and, moreover, allows neither a precise targeting of the tumors nor an increase in the lifetime of these carriers in the blood compartment. Specifically, the major drawback of the liposome-type system is its poor stability in the circulation due, in particular, to its rapid biodegradation by circulating enzymes.

Document No. US 5,527,528 describes the use of modified liposomes for carrying and administering anticancer medicinal products. The liposomes described are between 50 and 120 nm in size, contain an antitumor compound such as cytarabine and have, at their surface, molecular chains of poly(ethylene glycol) at a sufficient concentration to significantly increase the circulation time of said liposomes in the blood compartment of the host to which they are administered.

Moreover, these liposomes also carry, at their surface, antibodies specific for certain tumor antigens, allowing active targeting of the liposomes against the tumors.

That document discloses a carrier which provides both sustained transport of active principles in the circulation and a certain targeting to the type of tumor against which they are directed.

However, this type of carrier has a major drawback. Specifically, it is especially advantageous for anticancer agents which are hydrophilic in nature, such as cytarabine or gentamycin for example. Here again, the transport of lipophilic active principles, and in particular of anticancer agents such as daunomycin for example, is limited. These compounds are transported by incorporation into the double phospholipid layer of the liposomes. Because of this, the incorporation yield remains low since the volume of the core of the liposome is not used for the transport. The main drawback of these systems is represented by a limited capacity of incorporation for lipophilic medicinal products due to the unfavourable ratio of the aqueous compartment volumes to the lipophilic compartment volumes.

In addition, the membrane incorporation of active principles will depend on their degree of solubilization in the lipid environment of this phospholipid double layer. It is difficult to modulate the lipophilic nature within this bilayer given that the choice of the components thereof, in this case the phospholipids, is limited. This type of carrier does not therefore make it possible to adjust the HLB* (Hydrophilic Lipophilic Balance) of the transport medium with regard to the liposolubility of the active principle transported with as much ease as the core of the nanocapsules in accordance with the invention for which the choice of the constituents of the core offers greater versatility. The incorporation yields are therefore dependent on the degree of solubility of the active principle in the liposomal membrane. Thus, the liposomes are generally incapable of incorporating more than 5 mol% (most commonly 1 to 2%) of hydrophobic active principles into their membrane relative to all the lipids making up the composition of the lipid bilayer (Lasic, Trends Biotechnol. 16: 307, 1998).

*The HLB index, or hydrophilic/lipophilic balance, is as defined by C. Larpent in treatise K.342 of the publications "*Techniques de l'ingénieur*".

The liposomes sold under the trade mark Doxil[®] and intended to transport doxorubicin are also objects which exhibit certain stealth properties in the blood compartment. These liposomes have, at their surface, molecules of PEG inserted

into the phospholipid double layer, and thus provide passive targeting with respect to the tumors.

5 This active principle is "loaded" into the liposomes by a mechanism of "active loading" which results from the complexation of doxorubicin as it diffuses into the aqueous core. The pH gradient allows the doxorubicin to enter the liposomes, where this active principle will then change into the form of a gel. This filling mechanism allows a high active principle loading yield to be obtained.

10 However, this mechanism can only be exploited for certain active principles which are amphiphilic and ionisable in nature, which can readily diffuse across the lipid bilayer (therefore small molecules) and which have precipitation or complexing properties, such as doxorubicin or mitoxantrone, and is not applicable for most anticancer agents. The active principles which cannot benefit from this mechanism exhibit low loading yields and require subsequent steps of separation on a column and of recycling of the active principle.

15 In addition, the use of liposomes as carriers for active principles has other drawbacks. In particular, the presence of the phospholipid double layer physically limits the size of the liposomes obtained, which may, with difficulty, reach sizes of less than approximately 50 nm due to the limiting value of the radius of curvature which may be attained by this membrane.

20 The use of liposomal carriers therefore implies certain major drawbacks, in particular in their ability to transport several anticancer substances, but also with regard to the ease with which they can be used and stored.

25 Specifically, such liposomal carriers cannot be easily lyophilized without losing their structural properties and their capacity to transport when the colloidal solution is reconstituted. As a result, liposomal suspensions may exhibit quite poor stability and relatively short conservation times.

30 In order to attempt to overcome these drawbacks which limit the effectiveness of this type of carrier, nonliposomal colloidal carriers have been developed. Polymer-based micelles in fact offer a good alternative to liposomes in terms of transport of lipophilic active principles, and in particular of anticancer agents.

Thus, document No. WO 02/00194 describes a colloidal carrier in the form of polymer-based micelles. This carrier allows, inter alia, the transport and delivery of anticancer active principles such as doxorubicin or methotrexate for example. The micelles claimed comprise a hydrophobic central component into which a lipophilic anticancer agent can be inserted, and an outer "shell" which is hydrophilic in nature. These micelles in fact correspond to an assembly of diblock polymers comprising a hydrophilic half based on poly(vinylpyrrolidone) and a hydrophobic half consisting of poly(lactic acid). The hydrophobic central component corresponds to the coming together of the hydrophobic central components of the polymer molecules making up these micelles. This carrier, besides its ability to transport hydrophobic molecules, makes it possible to provide particles which are very small in size, and in particular less than 100 nm, which is difficult to achieve in the case of liposomes.

Other types of polymeric micelles have also been developed with the aim of vehiculing active principles in the blood for sustained times (Stolnik *et al.*, J. Drug Target, 9: 361, 2001). This type of carrier also has a structure of the diblock polymer type, the hydrophobic component consisting of poly(lactic acid) (PLA) and the hydrophilic components being represented by PEG. Depending on the length of each of the blocks, the PLA-PEG micelles obtained have various sizes, more generally around 75 nm. Intravenous injection of these micelles into rats reveals certain stealth properties with an elimination half-life of between 1 and 2 hours; 25% of the injected dose is still present in the blood after 3 hours.

On the other hand, the micelle-based carriers have the drawback that they are relatively unstable in the organism and can rapidly dissociate upon dilution. In fact, the stability of the micelles depends on the critical micelle concentration (CMC), below which the polymeric molecules which form them disassemble. This relative stability makes the industrial use of micelles for the therapeutic delivery of anticancer substances delicate.

Thus, carriers based on nanoparticles, other than liposomes, have been developed in order to overcome the lack of stability of the existing colloidal systems and/or their poor effectiveness in transporting molecules which are lipophilic in nature.

Thus, patent WO 01/64328 describes such lipid nanocapsules comprising a rigid lipid surface and a liquid or semi-liquid core composed of esters of fatty acids and of triglycerides. The rigid surface of these nanocapsules is composed, in part, of

Lipoid® S 75-3, which corresponds to a mixture of several lecithins, and of Solutol® HS 15. This type of carrier makes it possible to envisage the transport of hydrophobic active principles, and in particular of anticancer agents. In such nanoparticles, the lipophilic active principle is present in dissolved form inside the core, the composition of which may be modified so as to adjust the HLB as a function of the liposolubility of the active principle chosen. The lipid nanocapsules according to patent WO 01/64328 have been the subject of an evaluation of their behaviour in the blood *in vivo*, subsequent to the intravenous injection thereof into rats (Cahouet *et al.*, *13^{ème} Symposium International sur la Microencapsulation [13th International Symposium on Microencapsulation], Angers (France), Sept. 2001). Solutol® HS 15 which makes up the surface of these nanoparticles is a molecule consisting of a hydrophobic component and a short chain of poly(ethylene glycol) of 660 g/mol, protruding outside. As mentioned above, PEG chains which are so short, even at high density, are not sufficiently effective to confer on the nanoparticles a steric protection against opsonization and therefore stealth properties. Thus, the half-life time of these lipid nanocapsules is short since it is approximately 45 minutes. These lipid nanocapsules circulate, however, for a longer period of time than small molecules, such as radioactive labels alone. The half-life time of the lipid nanocapsules in accordance with patent WO 01/64328 would not therefore be sufficient to allow them to circulate for sustained periods of time in order to be able to accumulate in tumors (in organs other than the liver and the spleen), as mentioned previously.

Other types of lipid nanocapsules exhibiting, this time, a certain long circulation time in mice, and intended to transport anticancer active principles, have been produced and described by Mosqueira *et al.* (Pharm. Res., 18: 1411, 2001). Such lipid nanocapsules have both a core composed of fatty substances which allows lipophilic active principles, in particular anticancer agents, to dissolve and therefore to be transported, and a rigid outer layer of polymers onto which PEG chains are grafted, providing them with some stealth properties with respect to the immune system.

These nanocapsules, approximately 200 nm in diameter, exhibit a blood circulation time which is significantly greater than that of the same nanoparticles lacking said surface molecules. However, these results were obtained in mice, the immune system of which has a capacity for opsonization which is less than that

observed in rats or in humans for example. Their relative effectiveness in evading the immune system of mice does not therefore make it possible to extrapolate these results to the immune systems of rats or humans, which are more powerful in terms of capacity for opsonization (Liu D. *et al.*, BBA, 1240: 277, 1995).

5 Moreover, their relatively large diameter would not allow them to readily undergo extravasation in most solid tumors. Thus, even though the time of residence in the blood compartment is increased, the tumor targeting remains low, which limits all the more their therapeutic effectiveness.

10 Nanospheres composed of polymers made up of a diblock structure of PLA-PEG, exhibiting stealth properties in rats, with an elimination half-life of 6 hours, have also been developed (Verrecchia *et al.*, J. Control. Rel. 36: 49, 1995). The hydrophobic component of the polymer consists of PLA of 30 000 g/mol, and the hydrophilic chain is composed of PEG of 2 000 g/mol. These nanoparticles have a surface which is relatively unfavourable to complement activation, the consequence
15 of which is to decrease opsonization phenomena and phagocytosis by the cells of the MPS.

However, the major drawback of this type of nanoparticle based on solid polymers of the PLA type lies in their poor capacity to release the active principle rapidly, once in the area around the clump of tumor cells. Specifically, the rigid outer
20 layer of these nanocapsules is composed of PLA and is degraded relatively slowly by hydrolysis. This weak bioerosion implies a slow release of the active principles contained in these nanocapsules. Now, the therapeutic effectiveness of such anticancer carriers comes, firstly, from their ability to evade the immune system and, secondly, from their ability to rapidly release, i.e. in less than 24 hours, their content
25 in the area around the tumor. Now, the nanoparticles described by Mosqueira or Verrecchia are practically undegraded after having been in the organism for a period of 24 hours, which does not make it possible to envisage an optimal treatment of solid tumors.

The present invention thus provides a novel carrier for active principle(s),
30 which makes it possible both to avoid most of the drawbacks observed with regard to liposomal carriers, and to improve the effectiveness of targeting and of release of active principle(s) to/in the tumor compared to the existing nonliposomal colloidal carriers.

The aim of the present invention is also to provide a novel type of stealth

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carrier for active principle(s), which allows more effective treatment of cancers, in particular solid or circulating tumors.

Specifically, the nanocapsules according to the present invention, as a carrier for active principle(s), may be used for the treatment of solid tumors since the size and furtiveness characteristics of the nanocapsules in accordance with the invention allow these nanocapsules to approach and penetrate these tumors via a phenomenon of extravasation. In this case, reference is made to passive targeting of the tumors. Thus, to the applicant's credit, it has been able to design a carrier composed of stealth lipid nanoparticles allowing passive targeting of the tumors at which it is aimed.

In addition, the nanocapsules according to the present invention, as a carrier for active principle(s), may be used for the treatment of circulating tumors via a phenomenon of active targeting. This active targeting is produced by attaching, to the surface of said nanocapsules, molecules or "ligands" specific for certain surface molecules of the circulating tumors. The attachment of such surface molecules is advantageously carried out during the method for synthesizing the nanocapsules, by post-insertion, as described later. In general, the nanocapsules in accordance with the invention, as a carrier for active principle(s), may be used for the treatment of tumors which are solid or circulating in nature, by active targeting, when they have receptors which can be recognized by a ligand, or by passive targeting (without the involvement of a receptor).

The nanocapsules according to the present invention, as a carrier for active principle(s), also exhibit rapid and therefore more effective release of this (these) active principle(s) into the immediate environment of the tumor. Such a rapid release is permitted by the rapid bioresorption of the constituents of said nanocapsules, in particular due to the action of the enzymes present in the organism.

This characteristic of biodegradation, in particular by enzymatic digestion, is an additional advantage of the present invention, which makes it possible to improve the therapeutic effectiveness of the active principles administered in this way.

The carrier in accordance with the invention also exhibits advantageous stealth properties with respect to the immune system and complete biodegradability combined with a total lack of toxicity. In fact, all the constituents of the carrier in accordance with the invention are biodegradable and can be rapidly assimilated in

the organism, are all the subject of an approval for parenteral use and are also formulated without any organic solvent.

The novel stealth colloidal carrier in accordance with the invention also has the advantage of being readily lyophilizable and sterilizable, without losing its properties when it is reconstituted in the form of a suspension.

This carrier also makes it possible to administer, via the vascular route, medicinal products intended for the treatment of tissue inflammation.

The lipid nanocapsules according to the present invention advantageously have a diameter of between 50 and 150 nm, preferentially of between 80 and 120 nm.

The size of said nanocapsules plays a role in the passive targeting of the tumoral anticancer agents. Specifically, the EPR effect observed in the tumor allows the passage into, and the retention in, the interstitial medium which surrounds the solid tumor of elements present in the blood, the diameter of which is sufficiently small to allow this extravasation. The nanocapsules in accordance with the invention are prepared using a method which advantageously makes it possible to modulate their size. Moreover, they exhibit long circulating properties which provide them with a half-life time in the blood compartment of greater than two hours. These two characteristics advantageously make it possible to promote the extravasation phenomenon by increasing the number of passages made by the nanocapsules in the region of the fenestrations of the tumor capillaries. The stealth nature of the nanocapsules in accordance with the invention is also capable of promoting the contacts with malignant cells present in the blood circulation, in the case of circulating tumors.

Extravasation corresponds to a phenomenon of tangential filtration, which means that a tumor having pores of a diameter of 200 nm will allow circulating objects with the same diameter to pass only with difficulty. Moreover, the size of the nanocapsules in accordance with the invention allows them to avoid capture by extravasation at the level of the cells of the liver. In fact, the fenestrations of the sinusoidal capillaries of the liver allow the extravasation of particles of about 50 nm. Finally, said nanocapsules must be less than 200 nm in size in order to avoid the phenomenon of filtration observed in the spleen for particles greater than this in size. Thus, the defined size of the nanocapsules is responsible both for the phenomenon of passive targeting promoted by EPR, and for the increase in the

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amount of time said nanocapsules persist in the blood circulation by enabling them to avoid the phenomena of filtering and hepatic extravasation.

The diameter of the nanocapsules can be adjusted as a function of the type of tumor to be treated and may, where appropriate, be slightly less or more than the
5 abovementioned range.

The size of the nanocapsules must, in fact, be adjusted to the size of the fenestrations of the tumors to be destroyed, this size possibly varying over time, depending on the maturity of the tumor, and in space, within the same tumor. As a result, the passive targeting of the carrier in accordance with the invention can only
10 be envisaged on a therapeutic level if the size of the nanocapsules in accordance with the invention can be precisely adjusted. Now, the applicant has advantageously developed a method for precisely synthesizing stealth nanocapsules of variable and controllable size. The present carrier thus comprises abilities to adapt to the tumor environment which make it all the more effective.

15 The outer lipid envelope of the nanocapsules according to the present invention comprises a lipophilic surfactant which is lipidic in nature. This surfactant is advantageously in the form of a lecithin, the phosphatidylcholine proportion of which is at least equal to 95%, preferentially greater than 99%.

The applicant has noted that the constitution of nanocapsules comprising a
20 phosphatidylcholine content of greater than 95% makes it possible, in rats, to significantly increase the circulation time in the blood circulation. Thus, impurities such as other phospholipids, for instance phosphatidylethanolamine, lysophospholipids or free fatty acids, may contribute to increasing the phenomenon of opsonization of these nanoparticles. In addition, the applicant has also noted that
25 the yield of insertion of the molecules of lipid coupled to the molecules of PEG is better when the lipophilic surfactant exhibits an initial phosphatidylcholine concentration of greater than 95%.

Thus, the observed long circulation time of the nanocapsules according to the invention may come from the fact that the outer surface of these nanocapsules
30 consists of a practically pure phospholipid based on phosphatidylcholine, which limits opsonization. The degree of purity in terms of phosphatidylcholine plays a role in the mechanisms for avoiding opsonization.

The lipophilic surfactant which is lipidic in nature advantageously represents a molar percentage of between 5 and 30 mol% of the lipid molecules making up

said outer lipid envelope.

This surfactant preferably has a temperature of transition from gel to liquid phase of greater than 25°C, preferentially greater than 37°C; this means that, at ambient temperature, said surfactant is in gel form and only changes to liquid form at a temperature of greater than 25°C, preferentially greater than 37°C. The gel/liquid transition temperature plays a role in the rigidity of the outer lipid envelope of said nanocapsules. The rigidity of the outer envelope offers the nanocapsules according to the invention a considerable physicochemical stability, both in aqueous medium when they are in suspension, but also when they are administered in the blood compartment. In fact, the rigidity of said outer envelope confers on the nanocapsules a cohesion which allows them to withstand environmental modifications such as the variations in pH or in osmotic pressure which occur during the injection.

Advantageously, the lipophilic surfactant is a phospholipid carrying saturated carbon-based chains, preferably two chains, each of these chains comprising at least 16 carbon atoms. As a result of this, a phospholipid may preferably be chosen from C₁₈-C₁₈ DSPC (distearoylphosphatidylcholine), C₁₆-C₁₈ HSPC (hydrogenated soy phosphatidylcholine) and C₁₈-C₁₈ DPPC (dipalmitoylphosphatidylcholine). Preferentially, HSPC may be used at a degree of purity of greater than 99% so as to obtain better rigidity and better stealth properties. The latter compound is part of the composition of injectable liposomal formulations sold under the trade mark Doxil®.

Moreover, said outer lipid envelope comprises a hydrophilic surfactant which is lipid in nature, preferably approved for parenteral use. This hydrophilic surfactant is advantageously chosen from poly(ethylene glycol) alkyl esters and poly(ethylene glycol) alkyl ethers, and mixtures thereof.

This hydrophilic surfactant preferably represents between 60 and 90 mol% of the lipid molecules making up said outer lipid envelope. This surfactant is preferably used in a molar proportion of approximately 80 mol% of the lipid molecules making up the outer lipid envelope.

This hydrophilic surfactant is used, with the lipophilic surfactant, for the formation of the oil/water emulsion required for preparing the nanocapsules according to the invention. It in fact ensures stabilization of the fatty droplets formed from the mixture of triglycerides during the method for synthesizing said nanocapsules. Moreover, this surfactant also ensures the cohesion of said outer

lipid envelope. The relatively high proportion of hydrophilic surfactant in the envelope of said nanocapsules makes it possible to obtain a low interfacial tension, making it possible to prepare nanocapsules in which the higher the proportion of hydrophilic surfactant, the smaller the diameter.

5 Use will preferably be made of the nonionic surfactants sold under the names: Solutol® HS15 (BASF), Brij® (Uniquema) or Mapeg® (BASF). Use will preferentially be made of Solutol® HS15, which corresponds to poly(ethylene glycol)-660 12-hydroxystearate, comprising a short chain of 15 units of ethylene glycol corresponding to a molecular weight of 660 g/mol. This surfactant is
10 advantageously approved for parenteral use, and more particularly for intravenous administration; in addition, it gives good results in terms of cohesion.

 Finally, said outer lipid envelope comprises an amphiphilic derivative of PEG which contributes effectively to the long circulation time of said nanocapsules. Specifically, the amphiphilic derivative of PEG makes it possible at once to decrease
15 the phenomenon of opsonization of the nanocapsules, to improve the stability of the colloidal solution and to prolong the residence time for these nanocapsules in the blood circulation.

 The amphiphilic derivative of PEG advantageously comprises a hydrophobic component which allows it to be anchored in said outer lipid envelope and a
20 hydrophilic component of the PEG type facing the outside of said lipid nanocapsules, conferring hydrophilic properties at the surface thereof.

 The amphiphilic derivatives of PEG are inserted into the outer envelope of said nanocapsules via their hydrophobic end. Consequently, the PEG component faces the outside of said nanocapsules and forms a protective hydrophilic shell
25 surrounding each of the nanocapsules. The hydrophobic end allows anchoring at the surface of the outer envelope. This anchoring is provided by the low energy bonding forces which link the hydrophobic components of the pegylated lipids to those of the surfactants present at the surface of said lipid envelope. Specifically, the cohesion of the outer lipid envelope of the nanocapsules in accordance with the
30 invention results from the Van der Waals forces which are exerted between the hydrophobic groups, and in particular the alkyl chains of the molecules making up this envelope. These low energy bonds make it possible at once to maintain sufficient cohesion between the components of the envelope to avoid too rapid a degradation of the nanocapsules in accordance with the invention when they are

introduced into the blood compartment, but confer upon them, however, sufficient fragility to ensure rapid biodegradability and therefore a therapeutically effective release of the active principles transported.

In fact, unlike the polymeric chains of the PLA (poly lactic acid) or PLAGA (poly (lactio-co-glycolic acid)) type which constitute the surface of the nanocapsules as described by Mosqueira, and which are in solid form at any temperature under physiological conditions, the fatty chains which make up the envelope of the nanocapsules according to the invention are not covalently bound to one another, and are therefore more subject to disassembly. Specifically, bioerosion is a surface phenomenon which is the consequence of enzymatic attack or of the natural biodegradation (or bioresorption) linked to the local pH conditions and to the presence of water. This erosion is responsible for the loss of phospholipids at the surface of the nanocapsules which facilitate the exit of the active principles.

The polymer chains of the nanocapsules based on PLA or PLGA are long (45 000 g/mol for the nanocapsules described by Mosqueira) and constitute a thick surface which is both rigid and smooth. The biodegradation thereof is therefore all the slower. Thus, more than 24 hours (or even several weeks) are necessary for the biodegradation of the nanocapsules based on PLA or on PLGA to be complete. The phospholipids present at the surface of the nanocapsules according to the invention are distinct entities and advantageously allow extraction of the surface and rapid biodegradation, necessary for a therapeutically effective release of the active principles transported.

25 Finally, the constituents of the outer envelope of the nanocapsules are all not only biodegradable and approved for parenteral use and intravenous injection, but they can also be assimilated by the organism. Specifically, constituents such as phosphatidylcholine or phosphatidylethanolamine are commonly present in food and are assimilated by the organism naturally.

Thus, the size of the chains covering the surface of the nanocapsules, and also the nature thereof and the nature of the chemical bonds which keep them assembled, contribute to making the nanocapsules according to the invention both stable in the blood compartment for more than an hour and rapidly biodegradable and able to be assimilated by the circulating enzymes or enzymes present in the interstitial fluids.

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Advantageously, the half-life of the nanocapsules in accordance with the invention is greater than 2 hours. Ideally, for the nanocapsules in accordance with the invention, the intention will be to attain a plasmatic half-life of between 3 and 10 hours. This characteristic advantageously allows the nanocapsules according to the invention to accumulate in the tumors and to liberate therein most of their contents within reasonable amounts of time. The effectiveness of the anticancer agent therefore increases as a result. Now, the rapidity of release of the antitumor agent is a primordial factor to be taken into account for eliminating solid tumors, given their high rate of growth.

The amphiphilic derivatives of PEG used in the present invention preferentially have, as a hydrophobic group, a phosphatidylethanolamine. They are therefore pegylated phospholipids. Thus, use may be made of biodegradable phospholipids, in particular chosen from

DPPE-PEG_x (dipalmitoylphosphatidylethanolamine),
DSPE-PEG_x (distearoylphosphatidylethanolamine),
DOPE-PEG_x (dioleoylphosphatidylethanolamine), and
POPE-PEG_x (palmitoyloleoylphosphatidylethanolamine),

in which x represents the size of the PEG molecule in g/mol, and also mixtures thereof.

Preferentially, as a phospholipid coupled to a molecule of PEG, DSPE-PEG_x will be used for its qualities of stability in the outer envelope of the nanocapsules in accordance with the invention.

Advantageously, DSPE-PEG₂₀₀₀, DSPE-PEG₃₀₀₀ and DSPE-PEG₅₀₀₀ will be used.

Moreover, the stealth effect of the nanocapsules according to the present invention may also be obtained with pegylated lipids of the sterol nucleus-PEG, ceramide-PEG or PEG₂₀₀₀ sorbitan tetraoleate type, or more generally of the alkyl-PEG type having at least one alkyl chain providing hydrophobic anchoring to at least one molecule of PEG in the outer envelope.

The size of the molecule of PEG polymer grafted to the phospholipid described above is also of importance. Specifically, it will condition the spatial hindrance at the surface of said nanocapsules and, consequently, the accessibility to this surface but also the ability of these nanocapsules to evade the MPS.

The molecules of PEG of the amphiphilic derivatives used in the present

invention preferably have a molar mass of greater than or equal to 1 000 g/mol. As mentioned above, the presence of PEG chains less than 1 000 g/mol in size does not confer sufficient protection against opsonization. Consequently, the short chain of PEG of 660 g/mol present on the molecule of hydrophilic surfactant Solutol®

5 HS15 mentioned above could not confer effective protection of the surface against opsonization, and therefore sustained circulation. The molecules of PEG of formula $(O-CH_2-CH_2)_n$ which can be used in the context of the present invention belong to the group comprising PEG₆₀₀₀, comprising 113 PEG units, PEG₃₀₀₀, comprising 68 PEG units, and PEG₂₀₀₀, comprising 45 PEG units. These long pegylated chains

10 confer a hydrophilic nature at the surface of the lipid nanocapsules.

The molar proportion of the amphiphilic derivative of PEG integrated into the envelope of said nanocapsules is preferably between 0.5 and 12 mol% of the lipid molecules making up the outer lipid envelope, preferably between 1 and 10 mol%. Preferentially, the molecules of DSPE-PEG will be used in a molar proportion of

15 between 1 and 10 mol% of the total lipids of the envelope.

In a preferred embodiment, molecules of DSPE-PEG₅₀₀₀ will be used since their size (they are approximately twice as long as the molecules of DSPE-PEG₂₀₀₀) makes it possible, for the same number of molecules, to obtain greater repulsion via the steric effect and more significant stealth properties *in vivo*. Specifically, the PEG

20 chains are in constant movement at the surface of the lipid envelope, and thus, the longer they are the more they sweep a large space around the lipid nanocapsules, which increases the steric hindrance and decreases all the more the possibility for proteins to reach the surface of these nanocapsules. The ability to evade the phenomenon of opsonization is therefore greater in the presence of DSPE-PEG₅₀₀₀.

25 The core of the nanocapsules in accordance with the invention is an essentially lipid core composed of fatty acid esters and/or of triglycerides and/or oil as fatty substance. The triglycerides are advantageously chosen from medium chain triglycerides carrying from 6 to 14 carbon atoms and/or caprylic/capric triglycerides, and also mixtures thereof. More preferentially, use will be made, as a mixture of

30 fatty substances making up the core of the nanocapsules in accordance with the invention, of Labrafac® cc or Miglyol® 812 N which correspond to the following designations in the Pharmacopoeia: mixture of medium chain triglycerides, fractionated coconut oil or caprylic/capric triglycerides. These triglycerides may be used alone or as a mixture with other pharmaceutical oils or fatty acid esters which

are also the subject of an approval for parenteral use.

The fatty acid esters making up said essentially lipid core are advantageously chosen from the medium chain fatty acids carrying from 8 to 18 carbon atoms, such as ethyl palmitate, ethyl oleate, ethyl myristate, isopropyl myristate or octyldodecyl myristate, and also mixtures thereof.

Of course, the composition and the proportions of each of these constituents can be modulated and adjusted as a function of the hydrophilic/lipophilic balance (or HLB) desired for the mixture. In fact, since the inner face of the envelope is lipophilic in nature, there will be no incompatibility between the nature of the envelope and the content of the core.

These lipids are in liquid or semi-liquid form at ambient temperature depending on the size and on the degree of saturation of the carbon-based chains carried by the triglycerides and the oils constituting said core. The presence of fatty substances inside the core of the nanocapsules according to the invention contributes to acting as a solvent for the anticancer agents transported, but also constitutes a solid mass which is used for maintaining and for the relative stability of these nanocapsules in the external environment, whether this is in solution in an aqueous liquid or in the dry state at the end of a lyophilization step. Unlike the liposomes or the other colloidal carriers which have a lipid membrane and an aqueous interior, the presence of a fatty substance content therefore provides the nanocapsules according to the invention with a certain stability (variation in ionic strength or in osmotic pressure).

Advantageously, the outer surface of the outer lipid envelope of the stealth lipid nanocapsules which are the subject of the present invention is hydrophilic in nature and the essentially lipid core is lipophilic in nature.

Thus, the combination of biodegradable lipids which can be assimilated, which make up the core and the envelope, both makes it possible to envisage a rapid release of the active principles transported, and also provides the nanocapsules with a physical stability which allows them to be lyophilized and even sterilized by passing them through a 0.45 to 0.22 μm filter. The nanocapsules in accordance with the invention may therefore be sterilized and, optionally, stored in dry form for long periods of time. The novel carrier in accordance with the invention thus offers a certain ease of use which makes it a product which is readily exploitable in industry.

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Advantageously, the essentially lipid core of the stealth lipid nanocapsules which are subjects of the present invention represents between 20 and 60%, preferably between 25 and 50%, by weight relative to the total weight of said nanocapsules.

5 Advantageously, the aqueous phase or dispersant phase, in which the nanocapsules (or dispersed phase) in accordance with the invention may be prepared, initially contains a certain concentration of salts, advantageously of NaCl. This concentration is between 4 and 8%, advantageously within the range of 4.4 to 5.2%. At the end of the method of synthesis, osmotic agents, cryoprotective agents
10 or lyoprotective agents and, optionally, preserving agents may be added to this aqueous phase.

The applicant has in fact noted that this concentration of salt makes it possible at once to obtain a phase inversion temperature (or TIP) which is lower than in the absence of salts, to allow a more massive incorporation of phospholipid
15 molecules coupled to PEG and, finally, to significantly decrease the size of the nanocapsules obtained.

The fatty substance mixture constituting said core makes it possible to envisage carrying weakly to strongly lipophilic active principles, and more particularly anticancer substances. Specifically, depending on the liposolubility of
20 each active principle, the HLB will be adjusted to allow complete dissolution of the active principle. The present invention even allows the transport of a mixture of active principles of different solubility, for which an intermediate HLB will be applied, ensuring at least partial solubility of each anticancer agent.

Advantageously, the stealth lipid nanocapsules which are subjects of the
25 present invention contain one or more active principles, in particular which are lipophilic in nature.

Thus, the following anticancer active principles, which are mainly lipophilic in nature, may be used, alone or as a mixture: paclitaxel and derivatives thereof such as docetaxel, camptothecin and derivatives thereof such as irinotecan, topotecan or
30 rubitecan, busulfan, chlorambucil, phthalocyanins, carotenoids and daunomycin in particular.

Moreover, anticancer active principles which are amphiphilic in nature, such as cytarabin, cyclophosphamide, methotrexate, fluoro derivatives such as 5-fluorouracil or 5-fluorouridine, for example, or doxorubicin in particular, may also

be transported by the nanocapsules in accordance with the invention. The active principles will be dissolved beforehand in the fatty acid esters or the triglycerides or in pharmaceutical oils and fatty substances which are of injectable grade. Organic co-solvents, approved for parenteral administration, may be used in order to solubilize active principles which are poorly soluble in pharmaceutical oils; the solution obtained may then be mixed to these oils. The solutions of active principle thus prepared may be directly used in the method or may be the subject of subsequent dilutions in triglyceride mixtures, before they are used.

Finally, it is entirely possible to envisage the transport of any other type of active principle, and in particular of agents for treating inflammations or sites of infections in tissues. Thus, the nanocapsules in accordance with the invention, by virtue of their stealth properties and the fact that their size can be modulated, are particularly suitable for carrying and delivering medicinal products to tissues in which there is inflammation. Specifically, during inflammation, as observed in forms of arthritis for example, the fenestrations of the capillaries irrigating the inflamed area become enlarged so as to allow increased transport of certain elements present in the blood, in particular of proteins. The same is true of tissues in which there are sites of infection. As a result, even though the accumulation in these areas is less marked than in tumors, it is possible to envisage that the release of suitable active principles, in a region close to the inflammation or the infection, by nanocapsules in accordance with the invention makes it possible to reduce the consequences of such pathological conditions. Thus, it is entirely possible to envisage the transport, by the nanocapsules in accordance with the invention, of anti-inflammatory substances, such as ibuprofen, nonsteroidal anti-inflammatories such as indometacin or corticoids such as dexamethasone and also antibiotics, analgesics and anti-infectious agents such as amphotericin B.

The core of the nanocapsules in accordance with the invention is therefore composed of a fatty phase, the variable composition of which makes it possible to adjust the ability of the nanocapsules to solubilize a given anticancer agent.

1) Treatment of solid and circulating tumors

The present invention can be used, firstly, in anticancer therapies via the blood.

The nanocapsules in accordance with the invention may be used to treat solid tumors in humans.

Moreover, they may be used to treat circulating tumors of the leukaemia or lymphoma type. In fact, as has been mentioned above, the targeting of circulating
5 tumor cells can be obtained only if the particles of the colloidal system have a circulating time sufficient to promote encounters with the tumor cells before any capture by the liver or circulating MPS cells. Thus, the nanocapsules in accordance with the invention are particularly suitable for the treatment of circulating tumors.

These nanocapsules may also have capacities for active targeting by means
10 of the coupling of ligands having specific affinity for molecules of the receptor type located at the surface of the cancer cells.

Advantageously, the stealth lipid nanocapsules which are subjects of the present invention carry, at their surface, specific ligands which confer upon them the ability to actively target cells having receptors for these ligands, in particular tumor
15 cells.

Preferably, the ligand will be chosen so as not to stimulate, or to slightly stimulate, the phenomena of clearance of the particles.

Advantageously, said ligand is of the saccharide or oligosaccharide type or vitamin type, or alternatively of the oligopeptide, antibody fragment or monoclonal
20 antibody type.

The ligands of the saccharide or oligosaccharide type or vitamin type (folate, riboflavin), and even of the oligopeptide, antibody fragment or monoclonal antibody type, may advantageously be used. Thus, said ligands will be coupled, beforehand, to the reactive end of molecules of the activated pegylated phospholipid type or any
25 other lipid which may comprise a hydrophobic component, able to be anchored in the surface of the nanocapsules, a hydrophilic chain of the PEG type and a reactive chemical function at the end of this hydrophilic chain. These ligands, once attached to the end of these chains, will then preferentially be introduced using the post-insertion technique as described below. The post-insertion method in fact makes it
30 possible to introduce a particular type of ligand or ligands which are different in nature having diverse specificities capable of conferring a set of particular properties on the carrier in accordance with the invention.

2) Treatment of inflamed tissues (in particular arthritis) and infected

The use of the nanocapsules in accordance with the invention for the purpose of treating tissue infections and/or inflammations, in particular in humans, may also be envisaged. Specifically, as was described above, during tissue inflammations or infections, the capillaries irrigating the area subject to inflammation experience increased permeability. This temporary permeability can be exploited in the same way as for the treatment of solid tumors, using the phenomenon of extravasation to introduce into the inflamed tissue bioactive substances capable of decreasing or eliminating the causes and/or the consequences of the inflammation. Thus, it is possible to envisage, in particular, the transport, by the nanocapsules in accordance with the invention, of active principles intended to attenuate the inflammation, such as anti-inflammatories, analgesics or antibiotics for example. In the case of arthritis for example, which corresponds to inflammation of certain joints, an intra-articular injection of the nanocapsules in accordance with the invention will preferentially be given. This is because the targeting of the carrier directly to its site of action is thus promoted, and the loss by dilution of the nanocapsules in the entire blood circulation is, moreover, limited. Furthermore, the stealth properties of the nanocapsules in accordance with the invention should allow them not to be recognized and eliminated from the sites of inflammation by the large number of cells of the MPS and of the immune system present at these sites.

A subject of the present invention is also the pharmaceutical compositions comprising the nanocapsules according to the present invention, which contain one or more active principles. These pharmaceutical compositions are advantageously in the form of a colloidal aqueous suspension containing said nanocapsules.

Such a carrier may be administered parenterally, and in particularly intravenously, intra-arterially, intraperitoneally and intramuscularly, but it is also possible to envisage injecting it subcutaneously. In fact, subcutaneous injection will make it possible to reach the lymphatic vessels which may ensure accumulation of the nanocapsules according to the invention in the lymph nodes. This route of access is therefore particularly important for the treatment of lymph node ailments, and more particularly lymph node tumors.

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3) Detoxification

Advantageously, the lipid nanocapsules which are subjects of the present invention may be used to take up hydrophobic molecules present in the blood circulation subsequent to an instance of poisoning.

After instances of acute poisoning with an active principle which is hydrophobic in nature, stealth lipid nanocapsules which have not been loaded with an active principle may be administered intravenously. Thus, by virtue of their sustained circulation time and of their hydrophobic lipid core, the nanocapsules in accordance with the invention may take up the active principles present in the blood circulation so as to decrease the free concentration thereof and, consequently, the toxicity thereof. Subsequently, these active principles would be gradually released with the degradation of the carrier, at concentrations below the toxic free concentrations.

4) Sterilizing filtration

Finally, the nanocapsules according to the present invention have the advantage that they can be sterilized by sterilizing filtration through a filter with a diameter of 0.45 μm and even of 0.22 μm , this step possibly taking place directly after the final quenching step of the method described below. In fact, the applicant has noted that the nanocapsules according to the invention are sufficiently solid in suspension to be passed through this type of filter without undergoing any major modification and especially without losing their stealth properties and biodegradability. This characteristic allows said nanocapsules to be readily and safely employed for parenteral use, which makes them a carrier of choice for hospital-based or industrial mass use for example.

5) Use in dry/filtration form

The nanocapsules in accordance with the invention may advantageously be lyophilized and then reconstituted in the form of a colloidal suspension. They may thus be easily conserved for sustained periods of time. A suspension of nanocapsules according to the invention may in fact be reconstituted from a dry

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form extemporaneously, just before use. Such a lyophilization may take place directly after a sterilizing filtration step as described above.

5 The lyophilization of said nanocapsules consists in removing the water from the preparations by a phenomenon of sublimation. A suitable amount (approximately 5% m/m) of a cryoprotective or lyoprotective agent such as mannitol or trehalose, for example, may then be added to the suspension of nanocapsules to be lyophilized. After these compounds have completely dissolved, the suspensions chosen undergo a first step of rapid freezing at around -50°C. These suspensions are then lyophilized by direct passage of the water in the form of steam at low
10 temperature under reduced pressure. The nanocapsules in dry form may then be stored in sterile form for long periods of time before use.

6) Other administrations

15 It is possible to envisage using the nanocapsules in accordance with the invention, as a carrier for active principles, for treating disorders of types other than tumors or inflammations. Specifically, one of the qualities of this colloidal carrier is its ability to evade the host's immune system and in particular the cells of the MPS, such as macrophages. It is therefore possible to envisage using the stealth
20 nanocapsules of the invention in areas of the organism where the use of conventional carriers is particularly unsuitable. Such macrophage-rich areas exist in the pulmonary alveoli and the lymph nodes for example. It is thus possible to envisage administering said carrier via the routes for which phagocytosis and/or opsonization constitute a limit to the effectiveness of the pharmaceutical form.

25

METHODS FOR PREPARING THE NANOCAPSULES

The nanocapsules constituting the stealth colloidal carrier in accordance with the invention may advantageously be synthesized using two methods. The first
30 method is "conventional" since it is commonly used for synthesizing lipid nanocapsules. The second method has been developed by the applicant and is suitable for the nanocapsules according to the invention. It in particular makes it possible to obtain levels of incorporation of "pegylated" phospholipids which are greater than those obtained using the conventional method and also offers the

possibility of incorporating phospholipids coupled to ligands for providing active targeting. These two methods are detailed successively below.

5 1) CONVENTIONAL METHOD FOR SYNTHESIZING THE NANOCAPSULES

10 The method for synthesizing the stealth colloidal carrier in accordance with the invention is advantageously free of any organic solvent. Moreover, it is a simple and relatively rapid method which does not require any particular material. In addition, this method only uses biodegradable compounds approved for parenteral use and able to be naturally assimilated by the organism, via a phenomenon of "bioresorption" in particular. Finally, the method in accordance with the invention makes it possible to synthesize nanocapsules of defined diameter chosen by the user within a diameter range varying from 40 to 200 nm. Adjusting the size of the
15 nanocapsules synthesized will make it possible to adapt the carrier to the various types of tumor to be treated.

First of all, an oil/water emulsion is constituted from water, salt, the hydrophilic surfactant, the lipophilic surfactant, the fatty substances required for constituting the core of said nanocapsules, the molecules of lipids coupled to the
20 molecules of PEG and the active principle(s) intended to be transported by the nanocapsules according to the invention. The active principle will be dissolved beforehand in the oily fatty phase which must constitute the core of the nanocapsules in accordance with the invention.

25 The first step therefore consists in weighing all the constituents and in heating them to a temperature greater than the melting temperature of the lipophilic surfactant, with gentle stirring, for example magnetic stirring, until a homogeneous oil/water emulsion is obtained. The mixture is thus initially brought to 65°C when the lipophilic surfactant is represented by HSPC.

30 The phase inversion of this oil/water emulsion is then brought about so as to transform it into a water/oil emulsion. To do this, the temperature of the mixture is increased to a temperature T2 greater than the phase inversion temperature (TIP) of the mixture thus constituted. The temperature of the mixture is then decreased to a temperature T1.

The phase inversion may optionally be followed by the disappearance of the

conductivity of the formulation when the water/oil emulsion forms, but may generally be visible to the naked eye as described below. T1 is a temperature at which the conductivity is at least equal to 90-95% of the conductivity measured at 20°C. T2 is the temperature at which the conductivity disappears.

- 5 This operation is repeated by performing at least one cycle of temperature modification around the phase inversion zone between T1 and T2 until a translucent solution appears. Specifically, the organization of the system in the form of nanocapsules is reflected visually by a change in appearance of the initial system, which changes from opaque-white to translucent-white. This change occurs when
10 the temperature falls below the phase inversion temperature (TIP). This temperature is generally between 6 and 15°C below the TIP.

- 15 The number of cycles applied to the emulsion depends on the amount of energy required to form the nanocapsules. The phase inversion between the oil/water emulsion and the water/oil emulsion results in a decrease in the conductivity when the temperature increases, until it disappears. The mean
20 temperature of the phase inversion zone corresponds to the phase inversion temperature (TIP).

- 25 The oil/water emulsion may then be quenched, i.e. abruptly cooled, so as to obtain stable nanocapsules. This operation takes place with magnetic stirring, by
30 diluting the emulsion between 3- and 10-fold using deionized water at 2°C +/- 1°C cast rapidly into the fine emulsion. This quenching step makes it possible to take the nanocapsules from the emulsion form, for which the lipids which make them up are fluid, to the suspension form, for which they are in a more set and therefore structurally very stable state. The particles thus obtained are stirred for 5 minutes. In
35 the case of an industrial application, it is possible to envisage performing abrupt cooling of the preparation by circulating it in a system of thermal exchangers for example. In this particular case, dilution of the preparation is avoided.

- 40 Advantageously, the fatty phase is Labrafac® cc, the lipophilic surfactant is HSPC (hydrogenated soy phosphatidylcholine) and the hydrophilic surfactant is Soluto® HS15. The amphiphilic derivative of PEG is the pegylated phospholipid
45 DSPE-PEG₂₀₀₀ or -PEG₅₀₀₀. Under these conditions, T1 = 60°C, T2 = 85°C and the number of cycles is equal to 3.

In terms of the constituents of the envelope of the nanocapsules, the molar percentage of the lipophilic surfactant which is lipid in nature is between 5 and

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30 mol%. It is between 60 and 90 mol% for the surfactant which is hydrophilic in nature and between 0.5 and 5 mol% for the pegylated phospholipid.

An advantageous example of the molar percentage of the various constituents of the outer lipid envelope of said nanocapsules may be as follows:

- 5 • HSPC: 15.46 mol%
- Solutol® HS15: 81.2 mol%
- DSPE-PEG₂₀₀₀: 3.34 mol%

10 In this example, the concentration of salt of the initial emulsion is 4.4% (m/m), and the mass of Labrafac® cc constituting the core of the lipid nanocapsules represents 44% (m/m) of all the constituents which are lipid in nature or surfactants present in the preparation. This set of constituents will subsequently be referred to as total lipids (components other than water and sodium chloride); this term in particular includes the pegylated phospholipid. The ratio of the constituents of the outer lipid surface (mentioned above) to the total lipids is therefore 56%.

15 In general, the higher the mass ratio of the lipids making up the outer envelope of said nanocapsules to the total lipids constituting them, the smaller the nanocapsules. Thus, the size of the nanocapsules according to the invention can easily be modulated at the time they are synthesized, by choosing a suitable mass ratio of the lipids of the envelope to the total lipids.

20 Conversely, the size of the particles increases when the proportion of lipids constituting the core thereof increases.

 Moreover, the size of the particles decreases when the proportion of hydrophilic surfactant increases and when the proportion of (hydrophilic and lipophilic) surfactants increases. The surfactant causes a decrease in the interfacial tension and therefore stabilization of the system, which promotes the production of small particles. Specifically, the lower the interfacial tension, the more stable the suspension of lipid nanocapsules in an aqueous solution. For fixed amounts of hydrophilic and lipophilic surfactants and also of fatty acid esters, the addition of increasing amounts of pegylated lipids results in an increase in the size of the particles. It is possible to obtain various size ranges and various pegylated phospholipid contents by choosing given amounts of Solutol® HS15 and DSPE-PEG (reference should be made to Figure 1 of Example 4).

 According to a preferred embodiment, the fatty phase is Labrafac® cc, the lipophilic surfactant which is lipid in nature is HSPC (Northern Lipids), the nonionic

hydrophilic surfactant is Solutol® HS15 and the lipid coupled to the molecules of PEG is DSPE-PEG₂₀₀₀. These compounds have the following characteristics:

5 - Labrafac® cc (Gattefossé, Saint-Priest, France). It is an oil composed of caprylic/capric medium chain triglycerides (C₈ and C₁₀). The density thereof is 0.930 to 0.960 at 20°C. The HLB index thereof is about 1. The composition thereof is identical to Miglyol® 812N (Sasol, Germany). Labrafac® cc is included in the composition of preparations for parenteral nutrition.

10 - Solutol® HS15 (BASF, Ludwigshafen, Germany). It is a poly(ethylene glycol)-660 12-hydroxystearate. It therefore plays the role of hydrophilic surfactant in the formulation. It can be used by injection (in mice by IV LD 50 > 3.16 g/kg, in rats 1.0 < LD 50 < 1.47 g/kg).

15 - DSPE-PEG₂₀₀₀ (Northern Lipids Inc., Vancouver, Canada). It is N-(carbamoylmethoxy poly(ethylene glycol) 2000)-1,2-distearoyl-sn-3-phosphoethanolamine in the form of sodium salt. It confers on the lipid nanocapsules their stealth properties. The long chains of poly(ethylene glycol) make it possible to protect the surface of the nanocapsules, via their steric effect, by preventing the proteins of the blood circulation from being deposited thereon (opsonization). It can be used by injection and is included in the liposomal preparation Doxil® (Alza, Mountain View, USA).

20 The aqueous phase of the initial oil/water emulsion may also contain 4 to 8% of a salt such as sodium chloride. Modification of the salt concentration leads to a shift of the phase inversion zone. The higher the salt concentration, the lower the phase inversion temperature. This phenomenon is advantageous for the encapsulation of hydrophobic thermosensitive active principles. The incorporation thereof may then take place at a lower temperature.

25 The diameter of the nanocapsules is advantageously adjusted by adjusting the proportions of salt and of hydrophilic surfactant, and the purity of the lipophilic surfactant, in the starting mixture of the method of synthesis.

30 2) METHOD FOR SYNTHESIZING THE STEALTH NANOCAPSULES BY POST-INSERTION

The stealth lipid nanocapsules which are subjects of the present invention are advantageously prepared according to a method by post-insertion.

This method comprises a step of preformation of nanocapsules lacking amphiphilic derivative of poly(ethylene glycol), and then a step of post-insertion of said amphiphilic derivatives of poly(ethylene glycol) into the surface of these nanocapsules.

5 According to this method, said preformation step advantageously comprises the synthesis of nanocapsules lacking the amphiphilic derivative of PEG, according to the phase inversion of an oil/water emulsion brought about by several cycles of increase and decrease in temperature, as described above in the conventional method.

10 The method developed by the applicant is also free of any organic solvent and makes it possible to obtain a better yield of incorporation of "pegylated" phospholipids at the surface of the nanocapsules in accordance with the invention.

For example, it is possible to obtain nanocapsules in which the molar proportion of DSPE-PEG relative to the lipids of the envelope will be greater than
15 5 mol%, and preferentially of the order of 10 mol%. Such a proportion confers on the nanocapsules according to the invention good stealth properties. Now, it is difficult to obtain such proportions according to the conventional method of synthesis described above. Moreover, the method of synthesis by preformation of nanocapsules without PEG followed by the subsequent incorporation of DSPE-PEG
20 by insertion makes it possible to precisely adjust the size of the nanocapsules, and thus to adapt them to the type of tumors to be treated by adjusting the proportion and the length of the hydrophilic chains of the amphiphilic derivative. Besides the insertion of a greater proportion of molecules of DSPE-PEG, it is possible to envisage, using this post-insertion technique, to incorporate derivatives of
25 phospholipids comprising ligands at the end of a pegylated chain. These ligands may confer on the nanocapsules in accordance with the invention properties of active and specific targeting of cells of interest (solid and circulating tumors). This post-insertion method is particularly suitable for thermosensitive molecules since it is carried out at a temperature which does not exceed 60°C.

30 For example, nanocapsules based on HSPC, but lacking DSPE-PEG, are prepared beforehand in accordance with the method described above.

Said post-insertion step advantageously comprises a first step of coincubation of the preformed nanocapsules in the presence of the amphiphilic derivative of poly(ethylene glycol), and then a second "quenching" step during which

the amphiphilic derivative of poly(ethylene glycol)/preformed nanocapsules mixture thus obtained is abruptly cooled so as to reach a temperature of between 0 and 5°C.

For example, water is added to the preparation containing the nanocapsules lacking DSPE-PEG described above so as to bring it to a precise volume in a volumetric flask. Thus, the precise concentration of the lipids in mg/ml is known and may be used as a basis for calculating the amounts of DSPE-PEG₂₀₀₀ or -PEG₅₀₀₀, for example, to be added in order to attain, for example, 6 mol% relative to all the lipids (including DSPE-PEG) making up the composition of the outer envelope of the lipid nanocapsules of the invention. Advantageously, the preparation of nanocapsules and the micellar solutions of DSPE-PEG are, subsequently, brought to and maintained at, separately, 60°C for a period of 15 minutes.

The step of coincubation of the amphiphilic derivative of poly(ethylene glycol)/preformed nanocapsules mixture is carried out at a temperature very slightly higher than the gel/liquid phase transition temperature of said lipophilic surfactant which is lipid in nature, but lower than the phase inversion temperature of the amphiphilic derivative of poly(ethylene glycol)/preformed nanocapsules mixture, in order to avoid any disorganization of the system due to the phase inversion phenomenon. Referring back to the example above, it is noted that, at 60°C, the surface of the lipid nanocapsules is much more fluid and allows the insertion of additional phospholipids. The required volume of micellar solution of DSPE-PEG is then introduced into the preparation of nanocapsules. This is allowed to incubate for 1 h 30 at 60°C, stirring vigorously every quarter of an hour. At the end of this incubation period, the preparations are immersed in an ice bath for one minute so as to abruptly set the envelope of the nanocapsules and to trap therein the molecules of DSPE-PEG inserted.

This post-insertion method makes it possible to obtain nanocapsules in accordance with the invention, the DSPE-PEG content of which is higher than that which it is possible to attain using the conventional method. In fact, the nanocapsules thus prepared exhibit, *in vivo*, a much greater furtive nature compared with that which it is possible to obtain with the nanocapsules prepared according to the conventional method.

It is also possible to carry out the post-insertion of DSPE-PEG at lower temperatures (37-50°C) but for longer incubation times. Thus, this method of

insertion at low temperature may be suitable for the incorporation of derivatives of pegylated phospholipids which have, at the end of the PEG chain, thermosensitive groups such as certain oligopeptides and proteins for example. The latter compounds may be used as direction groups for the preparation of nanocapsules in accordance with the invention but allowing active targeting of tumor cells.

In addition, said post-insertion method makes it possible to introduce, in addition to the molecules of DSPE-PEG, activated phospholipids of the PDP-PEG₂₀₀₀-DSPE (pyridyldithiopropionylamino-PEG₂₀₀₀-DSPE) or MPB-PEG₂₀₀₀-DSPE (p-(maleimidophenyl)butaroylamino-PEG₂₀₀₀-DSPE) type (Northern Lipids Inc., Vancouver, Canada) which comprise reactive functions at the end of the pegylated chains. These reactive functions allow the coupling, once the nanocapsules according to the invention have been obtained, of compounds which are peptide in nature and which comprise thiol groups, such as oligopeptides or proteins of the antibody type, in order to confer on them active targeting properties.

FIGURES

In the examples described below, reference will be made to the following figures:

- Figure 1: Evolution of the size of nanocapsules prepared using HSPC, as a function of the molar percentage of DSPE-PEG₂₀₀₀
- Figure 2: Evolution of the size of nanocapsules prepared using HSPC, lacking DSPE-PEG, as a function of the surface lipids/total lipids ratio
- Figure 3: Evolution of the size of nanocapsules for various formulations obtained using the post-insertion method
- Figure 4: Comparison of blood profiles for formulations of nanocapsules prepared using DSPE-PEG₂₀₀₀ or DSPE-PEG₅₀₀₀
- Figure 5: Comparison of the stealth properties of various formulations of nanocapsules prepared using DSPE-PEG₂₀₀₀ or DSPE-PEG₅₀₀₀, or of nanocapsules prepared according to the prior art
- Figure 6: Comparison of the stealth properties of various formulations of nanocapsules prepared using the conventional method or using the post-insertion method

A) EXAMPLES OF PREPARATION OF STEALTH NANOCAPSULES ACCORDING TO THE CONVENTIONAL METHOD

In the following examples:

- 5 Mass % = mass percentage relative to the total weight of the nanocapsule
 Mol% = molar percentage relative to the outer envelope of the nanocapsule
 m%/env. = mass percentage relative to the weight of the outer envelope of the nanocapsule

10 Example 1: Preparation comprising DSPE-PEG₂₀₀₀

Production of a preparation comprising DSPE-PEG₂₀₀₀ at 2.86 mol% for a total of approximately 1 gram of lipids, according to the conventional method.

- 15 The constituents required for synthesizing the nanocapsules in accordance with the invention are introduced into a 20 ml scintillation vial, in the amounts given in Table 1.

Table 1

	Mass (mg)	Mass %	Number of moles	Mol%	m%/env.
HSPC	75	1.50%	9.843E-05	16.98%	13.25%
DSPE-PEG	46	0.92%	1.658E-05	2.86%	8.13%
Solutol HS15	445	8.90%	4.645E-04	80.15%	78.62%
Labrafac	504	10.08%			
NaCl	220	4.40%			
Water	3710	74.20%			
				Total lipids	1070
				Env./Total	52.90%

Total 5000 100.00%

- 20 In general, during weighing, maximum variations (relative to target values) of $\pm 1.5\%$ for the lecithin and the DSPE-PEG, of ± 0.2 to 0.5% for the hydrophilic surfactant (Solutol[®] HS15) and the triglycerides, and of $\pm 3\%$ for the salt, are permitted. The amount of water introduced is at least equal to the value given and at most greater than the value given by 2%. In fact, as was explained above, the sizes of the nanocapsules obtained depend mainly on the ratio of the sum of the lipids

constituting the envelope of the particles to the total mass of lipids (*Envelope/Total*).

After the weighing, the temperature is gradually increased to 60°C and the stirring is maintained for a few minutes, during which it is ensured that the aggregates of lecithin or of DSPE-PEG have disintegrated. The temperature is then increased to 80-85°C. Subsequently, the vial is transferred onto a non-heated plate and the temperature is allowed to drop, still with stirring, to 60°C. Three cycles of increase in temperature to 80°C and decrease to 60°C can then be undertaken. During the second increase in temperature, it is possible to observe the phase inversion phenomenon (below the phase inversion temperature (PIT), the solution is translucent, and it then becomes opaque and has a creamy appearance above the PIT).

At the end of the third cycle, quenching is carried out by adding a volume of cold water at 1°C (12.5 ml) when the temperature falls by 10°C below the temperature at which the change in appearance occurred, as observed during the second and during the third increase in temperature. Following the quenching, the stirring is maintained for a period of 5 minutes. In the example cited, the temperature at which the change in appearance occurred is 75°C, the maximum temperature to which the preparation was taken is 82°C and the quenching was carried out at 62°C.

Subsequently, analysis of the size distribution profile by dynamic light scattering may be carried out after filtration of the preparation through a filter with a porosity of 0.2 µm. The preparation cited as an example makes it possible to obtain nanocapsules in accordance with the invention which are 87.5 ± 14 nm in size. The DSPE-PEG₂₀₀₀ content is 2.86 mol% relative to all the constituents of the surface. The other proportions were reported previously in Table 1.

The maximum proportion of DSPE-PEG₂₀₀₀ which it is possible to attain using this conventional preparation method is 3.34 mol%. The addition of a further amount of this pegylated phospholipid results in the instability of the preparations obtained following the final quenching.

Example 2: Preparation comprising DSPE-PEG₅₀₀₀

Production of a preparation comprising DSPE-PEG₅₀₀₀ at 1.41 mol% for a total of approximately 0.6 gram of lipids, according to the conventional method.

The procedure is identical to that described in the previous example, with the exception of the amounts used, which are given in Table 2 below:

Table 2

	Mass (mg)	Mass %	Number of moles	Mol%	m%/env.
HSPC	37.5	1.50%	4.921E-05	15.54%	11.89%
DSPE-PEG	25.8	1.03%	4.461E-06	1.41%	8.18%
Solutol HS15	252	10.08%	2.630E-04	83.05%	79.92%
Labrafac	252	10.08%			
NaCl	116	4.64%			
Water	1816.7	72.67%			
				Total lipids	567.3
				Env./Total	55.58%

Total 2500 100.00%

For this preparation, the opaque/translucent change in appearance occurs around 72°C, the maximum temperature reached during the cycles is 81°C and the quenching was carried out below 65°C with a volume of 6 ml of iced water.

The mean size of the nanocapsules obtained, by dynamic light scattering, is 63.2 ± 9.83 nm after filtration of the preparation through a filter with a porosity of 0.2 μ m.

The maximum proportion of DSPE-PEG₅₀₀₀ which it is possible to attain using this conventional preparation method is 1.49 mol%.

Thus, the amount of phospholipids incorporated using the conventional method is all the more limited since these molecules have longer PEG chains.

B) Examples of production of nanocapsules according to the invention by post-insertion

Example 3: Preparation of nanocapsules according to the method of insertion of DSPE-PEG into preformed nanocapsules

Table 3 gives an example of amounts of the various constituents, making it possible to obtain formulations of lipid nanocapsules having diameters of around 80 nm, using the technique of insertion of DSPE-PEG into preformed nanocapsules.

The parent preparation of preformed nanocapsules without DSPE-PEG is prepared according to the conventional method. The amounts introduced are given in Table 3. After the quenching, the preparation is transferred into a volumetric flask and the volume is made up to 25 ml with distilled water.

Table 3

	Mass (mg)	Mass %	Number of moles	Mol%	m%/env.
HSPC	75	1.50%	9.843E-05	17.48%	14.42%
DSPE-PEG	0	0.00%	0.000E+00	0.00%	0.00%
Solutol HS15	445	8.90%	4.645E-04	82.52%	85.58%
Labrafac	504	10.08%			
NaCl	220	4.40%			
Water	3756	75.12%			

Total lipids	1024
Env./Total	50.78%

Total 5000 100.00%

Micellar solutions of DSPE-PEG₂₀₀₀ and DSPE-PEG₅₀₀₀ were prepared beforehand by weighing 75 mg of "pegylated" phospholipids, adding a sufficient amount of distilled water to produce dissolution and making the volume up, after total dissolution, to a precise total volume of 5 ml.

2-ml volumes of parent preparation are then distributed into stoppered haemolysis tubes which are placed in a water bath at 60°C. The micellar solutions are also placed in the water bath. The preparations of lipid nanocapsules and the micellar solutions of DSPE-PEG are preincubated separately at 60°C for 15 minutes. After this period of time, the required volumes are added so as to obtain a theoretical proportion of 6 mol% of phospholipids associated with molecules of PEG in the envelope of the nanocapsules. Thus, with the volumes and the concentrations of the micellar solutions given above, 532 µl of DSPE-PEG₂₀₀₀ or 1 108 µl of DSPE-PEG₅₀₀₀ are added. A control is also prepared by adding 800 µl of hot water.

After these amounts have been added, the solutions are left for 1 h 30 at 60°C and vortexed every quarter of an hour. After this incubation period, the preparations are plunged into an ice bath.

The sizes of the lipid nanocapsules obtained are given in Table 4:

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Table 4.

Preparation	Nanocapsule size (nm)
Nonincubated parent preparation	69 ± 12.9
Heated control	69.9 ± 14.2
DSPE-PEG ₂₀₀₀	77.9 ± 14
DSPE-PEG ₅₀₀₀	78.4 ± 13

The control preparation, incubated under the same conditions, has particles with a size close to those of the parent preparation not incubated at 60°C. The preparations having been incubated in the presence of DSPE-PEG exhibit a substantial increase in the mean diameter of the particles, suggesting the insertion of the "pegylated" phospholipids at the surface of the nanocapsules.

C) Influence of various parameters on the size of the nanocapsules in accordance with the Invention

Example 4: Control of the size of nanocapsules prepared according to the conventional method

The preparations are produced in accordance with the conventional method as described in Example 1. The preparations are produced with an amount of lipids of around 1 g. Figure 1 represents the evolution of the size of the nanocapsules, as a function of the proportion of DSPE-PEG₂₀₀₀ added, for amounts of hydrophilic surfactant, Solutol® HS15, fixed, respectively, at 414 mg (413.5 ± 0.50), 446 mg (445.85 ± 0.01) or 504 mg (504.4 ± 0.27). The amounts of HSPC, Labrafac® cc and NaCl are maintained constant for all the preparations, respectively at 76 mg, 504.5 mg and 220 mg.

In general, the size increases for decreasing amounts of Solutol® HS15. In the same series, for a given amount of Solutol® HS15, the size increases with the increasing proportions of DSPE-PEG added.

Example 5: Control of the size for nanocapsules prepared according to the post-insertion method

When the intention is to obtain particularly long circulation times or to introduce ligands, it is necessary to make use of the post-insertion method as described in Example 3. Thus, nanocapsules are generated beforehand, which are free of DSPE-PEG and for which it is possible to adjust the size by varying the mass ratio of the total of the lipids and surfactants constituting the surface of the particles to the total of all the lipids and surfactants making up the composition of the preparation (surface + core). The relationship between this surface lipid/total lipid ratio and the size of the nanocapsules obtained, suitable for the post-insertion method, is shown in Figure 2.

The formulations shown in Figure 2 were prepared for an amount of approximately 1 g of lipids. The amounts of HSPC, Labrafac[®] cc and NaCl are constant and correspond to those used in Example 4. The amounts of Solutol[®] HS15 used and the corresponding surface lipids/total lipid ratios (S/T ratio) are given in Table 5 below.

Table 5

Solutol [®] HS15 (mg)	S/T ratio
400.6	48.6%
413.2	49.2%
445.9	50.9%
504.2	53.5%

Subsequently, it is possible to introduce the pegylated phospholipid DSPE-PEG using the post-insertion method, in order to obtain the stealth nanocapsules in accordance with the invention. The post-insertion method is carried out according to Example 3 for a final total proportion of 6 mol% of DSPE-PEG 2000 or 5000.

The sizes obtained for the parent formulations (series SM), free of DSPE-PEG, having respectively 504, 446 and 401 mg of Solutol[®] HS15, and the daughter formulations in accordance with the invention, having been the subject of incorporation of DSPE-PEG 2000 and 5000 using said post-insertion method, are represented in Figure 3.

Thus, the insertion of DSPE-PEG₂₀₀₀ results in an increase in the diameter of

7.2 nm on average. The increase in size is more marked for the DSPE-PEG₅₀₀₀, which exhibits a mean increase of 9.1 nm in the diameter of the particles.

On the basis of these increases in diameter, which reflect the effective insertion of pegylated phospholipids at the surface of the lipid nanocapsules, and in the knowledge of the evolution of the size of the nanocapsules free of PEG as a function of the surface/total lipids ratio (Figure 2), it is possible to adjust the size of the nanocapsules in accordance with the invention so as to facilitate the extravasation through the fenestrations present in the endothelium of particular tumor capillaries.

D) Results of clearance experiments carried out *in vivo* with the stealth nanocapsules in accordance with the invention

Example 6: Kinetics of disappearance of the carrier (PEG₂₀₀₀/PEG₅₀₀₀ comparative data)

The blood clearance of the nanocapsules in accordance with the invention was evaluated in a comparative study between a formulation of nanocapsules based on 3.34 mol% of DSPE-PEG₂₀₀₀ ("2k-3.34" curve) and a formulation of nanocapsules based on DSPE-PEG₅₀₀₀, the molar proportion of DSPE-PEG₅₀₀₀ of which is 1.41 mol% ("5k-1.41"). These formulations were prepared according to said conventional method as described in Examples 1 and 2.

To do this, solutions of nanocapsules in accordance with the invention were injected intravenously into male rats (Sprague-Dawley, 325 g) in a proportion of 4 rats per formulation. The injected doses of lipids were chosen so as to be approximately 10 times less than the doses starting from which phenomena of "saturation of the MPS" may take place (which would result in sustained circulation times due to the capacity for purging being overrun). Thus, a solution of nanocapsules comprising a total amount of lipids of 2 mg in a volume of 400 μ l was injected into each rat.

The nanocapsules in accordance with the invention were labelled beforehand with a tritiated radioactive tracer: [³H]-cholesterol hexadecyl ether which is a nonexchangeable label. The dose of radioactive label represents 3 microCurie (μ Ci) per rat. Nine successive blood samples were taken after injection of the

nanocapsules, at 5, 15 and 30 minutes and then at 1, 2, 4, 8, 12 and 24 hours.

The blood samples are digested in the presence of Soluene 350 (Canberra Packard, Mississauga, Canada) for one hour at 50°C. After digestion, the blood samples are decolorized using 30% aqueous hydrogen peroxide. The scintillation fluid Hionic Fluor (Canberra) is then added and the samples are left to stand overnight. The amount of labelled nanocapsules present in the blood at the various times is then assayed by counting the radioactivity using a scintillation counter. Assaying controls makes it possible to determine the number of dpm (disintegrations per minute) corresponding to 100% of the dose. The total blood volume in rats is 75 ml/kg. Thus, it is possible to express the results as percentage of the total injected dose. The blood profiles obtained of this experiment are recapitulated in Figure 4.

Table 6 gives the name, the mean size and the molar proportion of DSPE-PEG incorporated, according to the conventional method, into the outer envelope of said lipid nanocapsules.

The nomenclature used reports the molar mass of the PEG component (2k, 5k for 2000 or 5000) and the molar percentage of DSPE-PEG relative to all the surface lipids.

Table 6: Description of the formulations used

Name	Size (nm)	DSPE-PEG
2k-3.34	77 ± 15	3.34 mol%
5k-1.41	61 ± 15	1.41 mol%

These results show that the plasmatic half-life of nanocapsules in accordance with the invention is between 2 and 3 hours in rats both for the nanocapsules based on DSPE-PEG₂₀₀₀ at 3.34 mol% and for the nanocapsules based on DSPE-PEG₅₀₀₀ at 1.41 mol%. However, it is important to note the greater stealth effect provided by the chains of DSPE-PEG₅₀₀₀. Specifically, for a number of moles which is more than two times less than the number of moles of DSPE-PEG₂₀₀₀ at the surface of the nanocapsules, the longer PEG chains in fact provide a protective effect comparable to that conferred by the chains having a mass of 2 000 g/mol.

Example 7: Comparative study of the nanocapsules of the invention compared with the nanocapsules of the prior art

A study, similar to that shown in Example 6, aimed at comparing the rates of disappearance in the blood of the lipid nanocapsules in accordance with the invention and of the lipid nanocapsules as described in the prior art in patent No. WO 01/64328, was carried out. The results obtained for formulations 1 to 4 were obtained in rats under conditions identical to the results given in Figure 4. The formulations represented are briefly described below and the data obtained are reported in Figure 5. This figure also comprises, by way of comparison, the data obtained by Stolnik *et al.* (available for 3 hours of blood kinetics only) and also by Cahouet *et al.*

Formulation 1: Conventional nanocapsules as described in patent No. WO 01/64328: the lipophilic surfactant is Lipoïd® S75-3, which is not a pure lecithin (70% of phosphatidylcholine) and the particles lack PEG;

Formulation 2: Nanocapsules based on Lipoïd® S75-3, but comprising DSPE-PEG₂₀₀₀ (1.65 mol%) introduced according to the conventional method;

Formulation 3: Nanocapsules based on HSPC (>99% of phosphatidylcholine) and on DSPE-PEG₅₀₀₀ (1.41 mol%), nanocapsules in accordance with the invention based on PEG₅₀₀₀ introduced according to the conventional method;

Formulation 4: Nanocapsules-DSPE-PEG₂₀₀₀ (3.34 mol%), nanocapsules in accordance with the invention based on PEG₂₀₀₀, maximum amount which can be incorporated according to the conventional method for HSPC;

FS: Stolnik formulation: micelles of PLA-PEG in the proportion 13:5 (the PEG component having a molar mass of 2 000 g/mol), the diameter of which is of the order of 75 nm. The data of the best formulation are taken from the graph contained in the published article (Stolnik *et al.*, J. Drug Target., 9: 361, 2001). These data are also obtained following intravenous injection into rats of an amount of nanocapsules which does not saturate the MPS;

NL A.A.: Conventional nanocapsules formulation of the prior art. These data were taken from the study by Cahouet *et al.* ("13^{ème} Symposium International sur la Microencapsulation [13th International Symposium on Microencapsulation]", Angers (France). Sept. 2001). These results were also obtained following intravenous

injection (unknown dose) into rats of lipid nanocapsules prepared by the abovementioned authors according to a formulation based on Lipoïd® S75-3 described in patent No. WO 01/64328.

Formulations 1 and 2 represent, respectively, formulations of conventional nanocapsules, the surface of which comprises Lipoïd® S 75-3 (lecithin comprising impurities), and these same nanocapsules, the surface of which also comprises 1.65 mol% of DSPE-PEG₂₀₀₀. This level is close to the maximum content which can be incorporated for nanocapsules comprising Lipoïd® S 75-3, prepared according to the conventional method. The DSPE-PEG₂₀₀₀ content of formulation 4 is also at its maximum by virtue of the conventional method. The lack of impurities and the homogeneity of the phosphatidylcholine HSPC make it possible to incorporate such DSPE-PEG contents into the nanocapsules in accordance with the invention (practically twice as much as with Lipoïd® S 75-3).

These results show that the nanocapsules in accordance with the invention (formulations 3 and 4) exhibit a significantly lower clearance than the lipid nanocapsules of the prior art (formulation 1, as described in patent No. WO 01/64328) which are very rapidly eliminated from the blood compartment. Thus, for formulation 1, only 10% of the injected dose is still present in the blood after one hour. The nanocapsules of the prior art therefore exhibit no stealth properties. Moreover, the incorporation of DSPE-PEG₂₀₀₀ into the nanocapsules of the prior art (formulation 2) only very slightly improved their persistence in the blood circulation: their half-life time is also less than one hour. In fact, the respective increases in the AUCs (Area Under the Curve between 5 minutes and 24 hours) relative to formulation 1 ($AUC_{Form\ 4}/AUC_{Form\ 1}$) are:

- 25 - Formulation 2: 1.09
- Formulation 3: 2.31
- Formulation 4: 2.68.

Moreover, as mentioned in the description of the prior art, the nanocapsules in accordance with patent No. WO 01/64328 have also been the subject of an *in vivo* study in rats by Cahouet *et al.* The kinetics of elimination from the blood of these nanocapsules exhibits a profile (formulation NL A.A. in Figure 5) which is slightly superior to that obtained for formulation 1 and relatively similar to that of formulation 2, probably due to the difference in injected dose (NL A.A.: unknown dose). The half-life time of the nanocapsules of Cahouet *et al.* (NL A.A.) is less than

one hour (precisely, 45 minutes). The profile obtained by the applicant for formulation 1 is thus relatively similar to that given in the study of the prior art. These two control formulations, which do not comprise any chain of PEG greater than 1 000 g/mol, show the ineffectiveness of the short PEG chains of Solutol® HS15 (660 g/mol) in conferring protection against rapid elimination from the blood circulation. Thus, these two formulations do not exhibit any stealth properties nor a sustained circulation (10% of the injected dose at the 2nd hour) and may be considered to be non-stealth references for evaluating the effect of the modifications to the composition of the carriers (use of a lecithin with high phosphatidylcholine purity), in particular the addition of long PEG chains.

Figure 5 also represents the data of the prior art as given in the publication by Stolnik *et al.* (legend FS). Their formulation of PLA-PEG micelles exhibits a stealth nature with slightly less than 40% of the injected dose still present in the blood circulation after two hours. However, the stealth nature of this colloidal preparation is less than that of the nanocapsules in accordance with the invention, which exhibit more than 50% and more than 60% of the injected dose after the 2nd hour for formulations 3 (DSPE-PEG₅₀₀₀) and 4 (DSPE-PEG₂₀₀₀), respectively. Given that the proportion of nanocapsules in accordance with the invention (formulations 3 and 4) present in the blood circulation during the first 4 hours following the injection is clearly greater than that of the stealth nanoparticles of the prior art (formulation FS), the amount of carrier liable to reach solid and/or circulating tumor cells will advantageously be increased, thus increasing the chances of success of the treatment.

Example 8: Potentiation of the stealth properties provided by the post-insertion method

An *in vivo* study, performed under the same conditions as the previous studies, was carried out with 4 different formulations. Thus, in order to evaluate the advantage of the post-insertion method, the applicant compared the stealth nature of three formulations prepared according to said post-insertion method, with the stealth nature of the best formulation prepared according to the conventional method. The four formulations which were the subject of this *in vivo* study are as follows:

Formulation 2k-3.34: Nanocapsules-HSPC-PEG₂₀₀₀ (3.34 mol%), nanocapsules in accordance with the invention based on PEG₂₀₀₀, maximum amount which can be incorporated according to the conventional method for HSPC. This formulation corresponds to formulation 4 of Example 7, injected into a new group of 4 rats;

Formulation 2k-6%: Nanocapsules based on HSPC having been the subject of incorporation of an amount of DSPE-PEG₂₀₀₀ corresponding to 6 mol% of the total lipids of the surface of the nanocapsules in accordance with the invention according to the post-insertion method, as detailed in Example 3;

Formulation 5k-6%: Nanocapsules based on the same parent formulation comprising HSPC, prepared for the production of the nanocapsules of formulation 2k-6%, but having been the subject of incorporation of DSPE-PEG₅₀₀₀ by the post-insertion method, nanocapsules in accordance with the invention;

Formulation 2k-10%: Nanocapsules based on the same parent formulation comprising HSPC, prepared for the production of the nanocapsules of formulation 2k-6%, but having been the subject of incorporation of 10 mol% of DSPE-PEG₂₀₀₀ by the post-insertion method, nanocapsules in accordance with the invention.

The results obtained for the elimination from the blood of the three formulations detailed above are given in Figure 6.

The data given in Figure 6 demonstrate the advantage of the post-insertion method. Specifically, said post-insertion method makes it possible to obtain nanocapsules in accordance with the invention, the stealth properties of which is clearly superior to that of nanocapsules in accordance with the invention but prepared according to the conventional method. Moreover, this study also demonstrates the stability and the conservation of the stealth properties, over time, of the nanocapsules in accordance with the invention. Formulation 2k-3.34 was prepared 3 months earlier and injected again for the present study. The profiles obtained are comparable (an amount slightly greater than 22% of the injected dose is found at the end of the 4th hour, as in Figure 4). The formulations shown in Figure 6 exhibit pronounced stealth properties. The formulations of control nanocapsules (without DSPE-PEG) or (formulation 1 of the preceding example and that of the study of the prior art) exhibit less than 10% of the residual dose after two hours.

The post-insertion method makes it possible to incorporate amounts of

DSPE-PEG which are greater than those which can be incorporated according to the conventional method, as shown by the blood profiles of Figure 6, which makes it possible to improve the circulation time of the preparations and to adapt them to applications requiring greater proportions of nanocapsules in the blood during the first 12 hours following the injection thereof. Thus, the formulations prepared according to said post-insertion method all exhibit approximately 60% of the injected dose at the end of the 4th hour. At the 8th hour, the residual dose of nanocapsules of formulation 2k-6% is more than 18%. The same proportion of 6 mol% of DSPE-PEG, but having longer PEG chains (DSPE-PEG₅₀₀₀) makes it possible to obtain more than 40% of the injected dose at the 8th hour. When more DSPE-PEG₂₀₀₀ is introduced, up to 10 mol%, it is possible to obtain a profile slightly superior to that of the formulation 5k-6%, with 50% of the injected dose at the 8th hour. The stealth properties of the formulations of nanocapsules in accordance with the invention prepared according to the post-insertion method is thus clearly greater than that observed for "stealth" solid nanoparticles as described in the literature.

CLAIMS

1. Stealth lipid nanocapsules which comprise an essentially lipid core which is liquid or semi-liquid at ambient temperature, and an outer lipid envelope comprising at least one hydrophilic surfactant which is lipidic in nature, at least one lipophilic surfactant which is lipidic in nature and at least one amphiphilic derivative of poly(ethylene glycol), wherein the molar mass of the poly(ethylene glycol) component is greater than or equal to 1 000 g/mol.
2. The stealth lipid nanocapsules according to Claim 1 wherein the molar mass of the poly(ethylene glycol) component is greater than or equal to 2 000 g/mol.
3. The stealth lipid nanocapsules according to Claim 1, wherein said lipophilic surfactant is a lecithin, the phosphatidylcholine proportion of which is at least equal to 95%.
4. The stealth lipid nanocapsules according to Claim 3, wherein said lipophilic surfactant is a lecithin, the phosphatidylcholine proportion of which is greater than 99%.
5. The stealth lipid nanocapsules according to Claim 1, wherein said lipophilic surfactant has a gel/liquid transition temperature of at least equal to 25°C.
6. The stealth lipid nanocapsules according to Claim 5, wherein said lipophilic surfactant has a gel/liquid transition temperature greater than 37°C.
7. The stealth lipid nanocapsules according to Claim 1, wherein the lipophilic surfactant is a phospholipid comprising acyl chains of at least 16 carbon atoms.
8. The stealth lipid nanocapsules according to Claim 7, wherein said lipophilic surfactant is selected from the group consisting of HSPC (hydrogenated soy phosphatidylcholine), DSPC (distearoylphosphatidylcholine) and DPPC (dipalmitoylphosphatidylcholine), and also mixtures thereof.

9. The stealth lipid nanocapsules according to Claim 1, wherein said lipophilic surfactant represents between 5 and 30 mol% of the molecules making up said outer lipid envelope.

5

10. The stealth lipid nanocapsules according to Claim 1, wherein said hydrophilic surfactant is selected from the group consisting of poly(ethylene glycol) alkyl esters and poly(ethylene glycol) alkyl ethers, and also mixtures thereof.

10

11. The stealth lipid nanocapsules according to Claim 10, wherein said hydrophilic surfactant is a nonionic surfactant of the poly(ethylene glycol)-660 12-hydroxystearate type comprising a chain of 15 units of ethylene glycol.

15

12. The stealth lipid nanocapsules according to Claim 1, wherein said hydrophilic surfactant represents between 60 and 90 mol% of the molecules making up said outer lipid envelope.

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13. The stealth lipid nanocapsules according to Claim 12, wherein said hydrophilic surfactant represents 80 mol% of the molecules making up said outer lipid envelope.

25

14. The stealth lipid nanocapsules according to Claim 1, wherein said amphiphilic derivative of poly(ethylene glycol) comprises a hydrophobic component which allows it to be anchored in said outer lipid envelope and a hydrophilic component of the poly(ethylene glycol) type facing the outside of said lipid nanocapsules, conferring hydrophilic properties at the surface thereof.

30

15. The stealth lipid nanocapsules according to Claim 1, wherein said amphiphilic derivative of poly(ethylene glycol) is chosen from biodegradable phospholipids.

16. The stealth lipid nanocapsules according to Claim 15, wherein said biodegradable phospholipids are selected from the group consisting of DPPE-PEG_x (dipalmitoylphosphatidylethanolamine),

DSPE-PEG_x (distearoylphosphatidylethanolamine),
DOPE-PEG_x (dioleoylphosphatidylethanolamine), and
POPE-PEG_x (palmitoyloleoylphosphatidylethanolamine),
in which x is greater than or equal to 1 000 g/mol, and also mixtures thereof.

5

17. The stealth lipid nanocapsules according to Claim 16, wherein said biodegradable phospholipids are selected from the group consisting of DSPE-PEG₂₀₀₀, DSPE-PEG₃₀₀₀ and DSPE-PEG₅₀₀₀, and also mixtures thereof.

10

18. The stealth lipid nanocapsules according to Claim 1, wherein said amphiphilic derivative of poly(ethylene glycol) represents between 0.5 and 12 mol% of the molecules making up said outer lipid envelope.

15

19. The stealth lipid nanocapsules according to Claim 18 represent between 1 and 10 mol% of the molecules making up said outer lipid envelope.

20

20. The stealth lipid nanocapsules according to Claim 1, wherein said essentially lipid core represents between 20 and 60% by weight relative to the total weight of said nanocapsules.

21. The stealth lipid nanocapsules according to Claim 20 represent between 25 and 50% by weight relative to the total weight of said nanocapsules.

25

22. The stealth lipid nanocapsules according to Claim 1, wherein said essentially lipid core is composed of fatty acid esters and/or of triglycerides and/or of oil.

30

23. The stealth lipid nanocapsules according to Claim 22, wherein the triglycerides making up said essentially lipid core are chosen from the medium chain triglycerides carrying from 6 to 14 carbon atoms, caprylic/capric triglycerides, and mixtures thereof.

24. The stealth lipid nanocapsules according to Claim 22, wherein the fatty acid esters making up said essentially lipid core are selected from the group

consisting of medium chain fatty acids carrying from 8 to 18 carbon atoms.

25. The stealth lipid nanocapsules according to Claim 24 are selected from the group consisting of ethyl palmitate, ethyl oleate, ethyl myristate, isopropyl myristate, octyldodecyl myristate, and mixtures thereof.

26. The stealth lipid nanocapsules according to Claim 1, being between 50 and 150 nm, in diameter.

10 27. The stealth lipid nanocapsules according to Claim 26 being 80 and 120 nm, in diameter.

28. The stealth lipid nanocapsules according to Claim 1, wherein the outer surface of said outer lipid envelope is hydrophilic in nature, and the essentially lipid core is lipophilic in nature.

29. The stealth lipid nanocapsules according to Claim 1, carrying at their surface specific ligands which confer upon them the ability to actively target cells having receptors for these ligands, in particular tumor cells.

20 30. The stealth lipid nanocapsules according to Claim 29, wherein said ligand is selected from the groups consisting of the saccharide, oligosaccharide, vitamin, oligopeptide, antibody fragment and monoclonal antibody type.

25 31. The stealth lipid nanocapsules according to Claim 1, having a half-life of at least 2 hours in the blood compartment of the host to which they are administered.

30 32. The stealth lipid nanocapsules according to Claim 1, being able to rapidly release most of their contents by biodegradation, and in particular by enzymatic digestion.

33. The stealth lipid nanocapsules according to Claim 1, containing one or more active principles.

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34. The stealth lipid nanocapsules according to Claim 33, containing one or more anticancer active principles which are mainly lipophilic in nature.

5 35. The stealth lipid nanocapsules according to Claim 34 wherein the anticancer active principles are selected from the group consisting of paclitaxel and derivatives thereof, such as docetaxel, camptothecin and derivatives thereof, such as irinotecan, topotecan, rubitecan, and busulfan, chlorambucil, phthalocyanins, carotenoids and daunomycin.

10 36. The stealth lipid nanocapsules according to Claim 33, containing one or more anticancer active principles which are amphiphilic in nature.

15 37. The stealth lipid nanocapsules according to Claim 33 wherein the anticancer active principles are selected from the group consisting of cytarabine, cyclophosphamide, methotrexate, fluoro derivatives, such as 5-fluorouracil or 5-fluorouridine, and doxorubicin.

20 38. The stealth lipid nanocapsules according to Claim 33, containing one or more active principles selected from the group consisting of anti-inflammatories, corticoids, antibiotics, analgesics and anti-infectious agents.

25 39. The stealth lipid nanocapsules according to Claim 38 containing dexamethasone, indomethacin or amphotericin B.

30 40. A method for preparing the nanocapsules according to Claim 1, comprising preforming nanocapsules lacking amphiphilic derivative of poly(ethylene glycol), and then post-inserting said amphiphilic derivatives of poly(ethylene glycol) into the surface of these nanocapsules.

41. The method according to Claim 40, wherein said preformation step comprises the synthesis of nanocapsules lacking amphiphilic derivative of poly(ethylene glycol), according to the phase inversion of an oil/water emulsion brought about by several cycles of increase and decrease in temperature.

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42. The method according to Claim 40, wherein said post-insertion step comprises a first step of coincubation of the preformed nanocapsules in the presence of the amphiphilic derivative of poly(ethylene glycol), and then a second "quenching" step during which the amphiphilic derivative of poly(ethylene glycol)/preformed nanocapsules mixture thus obtained is abruptly cooled so as to reach a temperature of between 0 and 5°C.

43. The method according to Claim 42, wherein the step of coincubation of the amphiphilic derivative of poly(ethylene glycol)/preformed nanocapsules mixture is carried out at a temperature very slightly higher than the gel/liquid phase transition temperature of said lipophilic surfactant which is lipid in nature, but lower than the phase inversion temperature of the amphiphilic derivative of poly(ethylene glycol)/preformed nanocapsules mixture.

44. The method according to Claim 40, wherein the diameter of the nanocapsules is adjusted by adjusting the proportion and the length of the hydrophilic chains of the amphiphilic derivative when it is introduced in the post-insertion step.

45. A method for preparing the nanocapsules according to Claim 1, wherein the diameter of the nanocapsules is adjusted by adjusting the proportions of salt and of hydrophilic surfactant, and the purity of the lipophilic surfactant, in the starting mixture of the conventional method of synthesis.

46. The method according to Claim 40 or 45, being free of any organic solvent and using only biodegradable compounds approved for parenteral use.

47. The method according to Claim 40 or 45, wherein said nanocapsules are sterilized by sterilizing filtration through a filter with a diameter of 0.45 μm to 0.22 μm .

48. The method according to Claim 40 or 45, wherein said nanocapsules are lyophilized and then reconstituted extemporaneously in the form of a colloidal

suspension.

49. A pharmaceutical composition comprising the lipid nanocapsules according to Claim 1.

50. The pharmaceutical composition according to Claim 49, being in the form of a colloidal aqueous suspension containing said lipid nanocapsules.

51. A method for treating cancer comprising the intravenous administration of the lipid nanocapsules according to Claim 34 or Claim 36, to a subject in need thereof.

52. The method according to Claim 51, for treating solid or circulating tumors.

53. The method according to Claim 52, for treating circulating tumors by active targeting.

54. The method according to Claim 51, for treating solid tumors by passive targeting subsequent to the extravasation of said nanocapsules through the tumor capillaries.

55. A method for treating inflammations and/or infections of tissues comprising administering to a subject in need thereof the lipid nanocapsules according to claim 38.

56. The method according to Claim 51 or Claim 55, wherein the medicinal product is intended to be administered parenterally.

57. The method according to Claim 56, wherein the medicinal product is injected into the circulation of a subject intravascularly, intravenously, intra-arterially, intraperitoneally and intramuscularly, subcutaneously and intra-articularly.

58. A method for taking up hydrophobic molecules present in the blood

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circulation of a subject subsequent to an instance of poisoning comprising administering to said subject lipid nanocapsules according to Claim 1.

ABSTRACT

The present invention relates to stealth lipid nanocapsules consisting of an essentially lipid core which is liquid or semi-liquid at ambient temperature, and an outer lipid envelope comprising at least one hydrophilic surfactant which is lipidic in nature, at least one lipophilic surfactant which is lipidic in nature and at least one amphiphilic derivative of poly(ethylene glycol), the molar mass of the poly(ethylene glycol) component of which is greater than or equal to 1 000 g/mol, preferably greater than or equal to 2 000 g/mol, to the methods for the preparation thereof and to the use thereof as a carrier for active principle(s).

" STEALTH LIPID NANOCAPSULES, METHODS FOR THE PREPARATION THEREOF AND USE THEREOF AS A CARRIER FOR ACTIVE PRINCIPLE(S) "

**COMBINED DECLARATION AND POWER OF ATTORNEY
FOR UTILITY PATENT APPLICATION**

Attorney's Docket No.

017753-167

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (if only one name is listed below) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (if more than one name is listed below) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED:

STEALTH LIPID NANOCAPSULES, METHODS FOR THE PREPARATION THEREOF

AND USE THEREOF AS A CARRIER FOR ACTIVE PRINCIPLE(S)

the specification of which

(check one)

☐

is attached hereto;

☒

was filed on SEPTEMBER 9, 2002 as

Application No. _____

and was amended on _____;
(if applicable)

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE;

I ACKNOWLEDGE THE DUTY TO DISCLOSE TO THE OFFICE ALL INFORMATION KNOWN TO ME TO BE MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS, Sec. 1.56 (as amended effective March 16, 1992);

I do not know and do not believe the said invention was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said application; that said invention was not in public use or on sale in the United States of America more than one year prior to said application; that said invention has not been patented or made the subject of an inventor's certificate issued before the date of said application in any country foreign to the United States of America on any application filed by me or my legal representatives or assigns more than twelve months prior to said application;

I hereby claim foreign priority benefits under Title 35, United States Code Sec. 119 and/or Sec. 365 of any foreign application(s) for patent or inventor's certificate as indicated below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application(s) on which priority is claimed:

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Attorney's Docket No.

017753-167

COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
FRANCE	02/07175	11 JUNE 2002	YES <u>X</u> NO <u> </u>
			YES <u> </u> NO <u> </u>

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RESIDENCE	CITIZENSHIP	

APPL. FILING DATE: SEPTEMBER 9, 2002

TITLE: STEALTH LIPID NANOCAPSULE;

THE PREPARATION THEREOF AND USE THEREOF

CARRIER FOR ACTIVE PRINCIPLE(S)

INVENTOR(S): DIDIER HOARAU ET AL.

APPLICATION SERIAL NO:

SHEET 1 of 3

1/3

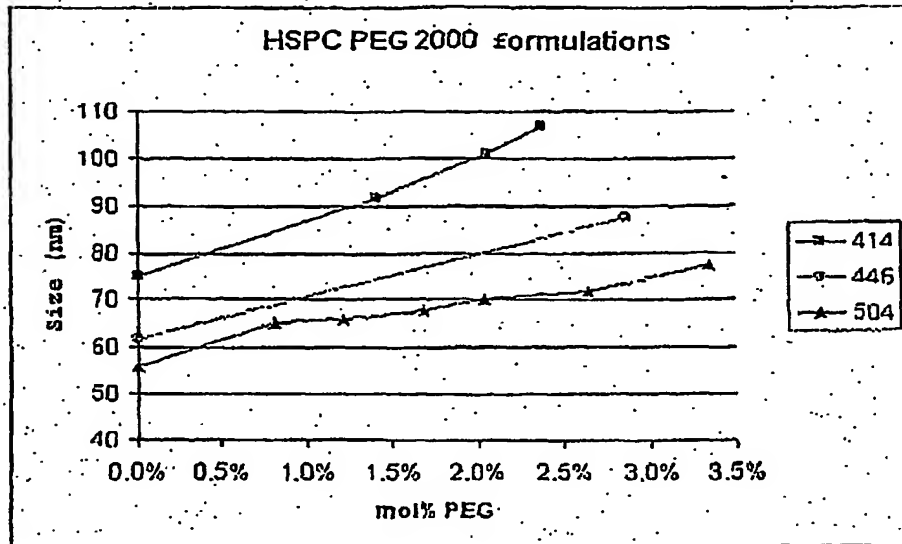


Figure 1

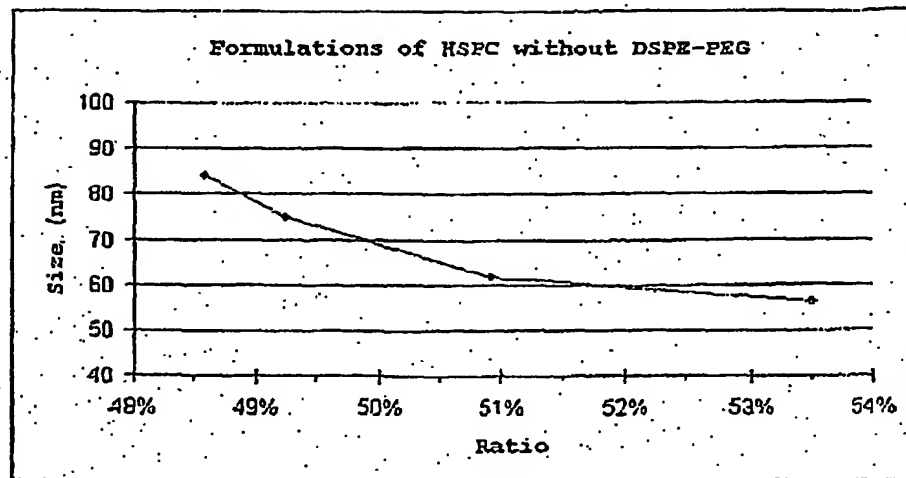


Figure 2

APPLN. FILING DATE: SEPTEMBRE

TITLE: STEALTH LIPID NANOCAPSULES

THE PREPARATION THEREOF AND USE THEREOF AS A CARRIER FOR ACTIVE PRINCIPLE(S)

INVENTOR(S): DIDIER HOARAU ET AL.

APPLICATION SERIAL NO:

SHEET 2 of 3

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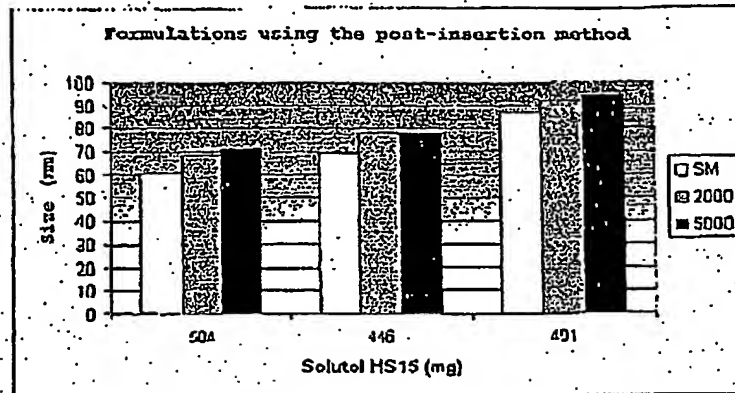


Figure 3

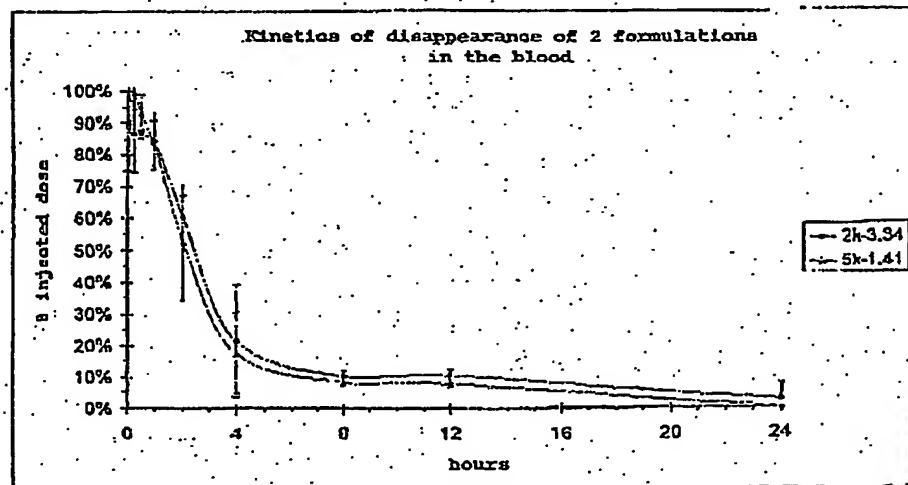


Figure 4

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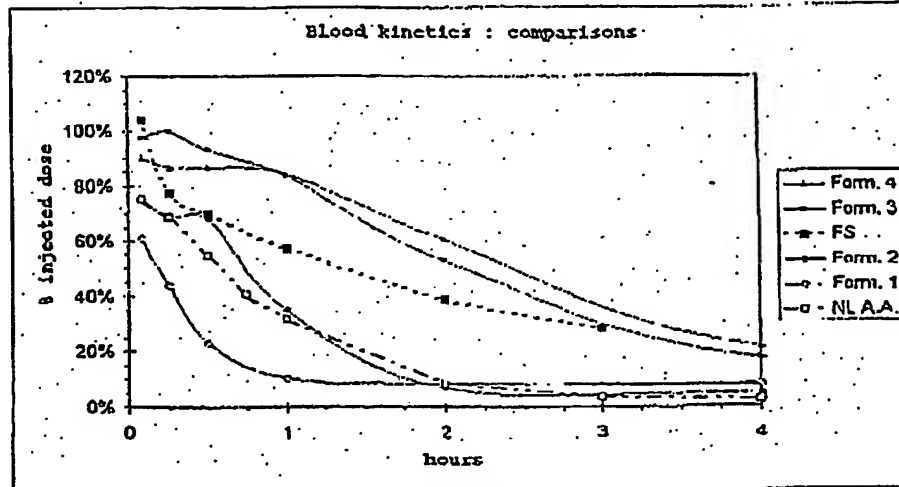


Figure 5

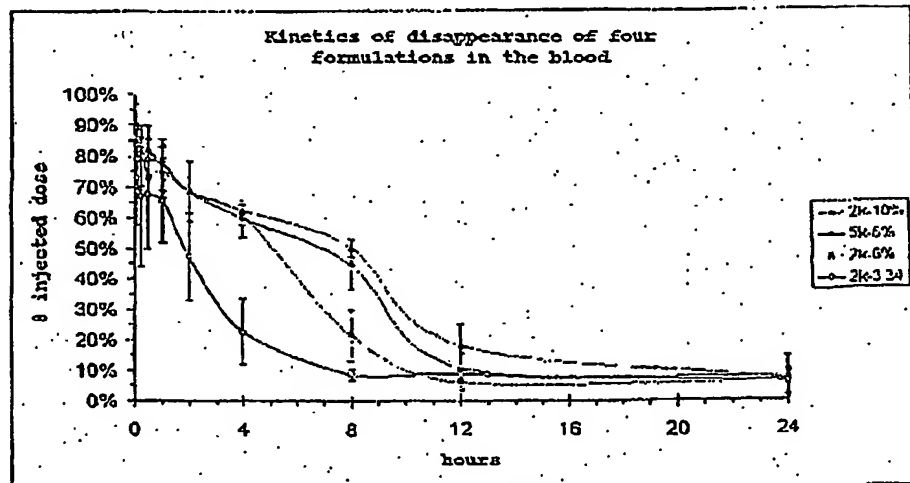


Figure 6

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